Spectroscopies vibrationnelles (MCR et ATR-FTIR) et Chromatographie Liquide couplée à la Spectrométrie de Masse Haute Résolution (LC-HR-MS) : Outils d'investigation in vivo de l'impact du vieillissement cutané sur le Stratum Corneum aux niveaux tissulaire, supra-moléculaire et moléculaire

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THÈSE DE DOCTORAT

CHIMIE

par

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et

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tissulaire, supra-moléculaire et moléculaire

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|RÉSUMÉ GÉNÉRAL

La peau est l'organe le plus étendu du corps humain. Doté d'une membrane biologique fine appelée la couche cornée, celle-ci le protège du desséchement et des agressions extérieures chimiques ou mécaniques auxquelles le corps humain doit faire face. Ce travail de thèse a consisté, dans un premier temps, à décrire via la littérature existante les effets de l'âge, dûs au vieillissement intrinsèque et extrinsèque, sur la physiologie cutanée du *Stratum Corneum* (SC). La partie expérimentale basée sur la microscopie vibrationnelle traitera des variations de la fonction barrière et de l'hydratation du SC lors du vieillissement chronologique et photo-vieillissement. D'autres méthodes ont également été utilisées comme la chromatographie liquide en phase normale couplée à la spectrométrie de masse haute résolution (CL-NP-SM-HR) pour l'étude de la composition détaillée des lipides du SC ainsi que des méthodes plus globales comme la PIE ou la conductance. Le caractère non invasif de toutes ces méthodes a permis de réaliser ces études *in vivo.* L'évolution de la fonction barrière a été étudiée aux niveaux tissulaire, moléculaire et supramoléculaire par micro-spectroscopie confocale Raman et spectroscopie infrarouge. Puis le lien moléculaire a été fait entre le vieillissement intrinsèque et les céramides de la matrice lipidique intercornéocytaire par CL-SM-HR. Les molécules discriminantes entre population jeune et âgée ont été déterminées par analyse chimiométrique. L'évolution de l'hydratation cutanée aux niveaux tissulaire, moléculaire et supramoléculaire a également été l'objet d'une investigation approfondie. Les variations de la composition des NMF et la teneur en eau dans le SC lors du vieillissement cutané ont été mises en lumière en utilisant des descripteurs spectraux Raman. Les variations structurelles des molécules d'eau impactant l'organisation supramoléculaire des édifices lipidiques ont également été évaluées. Le vieillissement chronologique et l'exposition chronique à des facteurs environnementaux affectent légèrement la fonction barrière du SC et l'hydratation. Cependant, les processus qui contrôlent ces propriétés sont affectés par le vieillissement, selon le site corporel.

|SUMMARY

Skin is the external surface defining the human body in space. Its outer-most layer is a thin biological membrane, called *Stratum Corneum*(SC), that protects the internal organs from desiccation as well as chemical or mechanical external aggressions. The present thesis aims in a first step, to summarize the current knowledge regarding the effects of intrinsic and extrinsic aging on SC physiology, based on available literature. The experimental part addresses the gaps in our understanding of the effects of chronological aging and photoaging on the SC barrier function and hydration, using traditional methods (such as Trans Epidermal Water Loss and skin conductance) as well as more advanced ones (vibrational spectroscopies, liquid chromatography in normal phase tandem mass spectrometry high resolution with an APCI source and an Orbitrap detector. As these methods are non-invasive, all studies have been carried out *in vivo*. The evolution of the barrier function has been studied at the tissular, molecular and supramolecular levels using confocal Raman micro-spectroscopy and infrared spectroscopy. Then the link between the intrinsic aging and the ceramides of the intercorneocytary lipid matrix has been studied by liquid chromatography tandem mass spectrometry. The discriminant molecules between young and old population have been identified by a chemometric analysis. The evolution of cutaneous hydration at the tissular, molecular and supramolecular level has also been investigated. The variations in the NMF composition and the SC water content have been studied by Raman spectral descriptors. Moreover, the structural variations of water molecules impacting the supramolecular organization of the lipid structures have been evaluated. Chronological aging and chronic exposure to environmental factors mildly affect SC barrier function and hydration levels. However, the processes controlling these properties are affected by aging in a site-dependent fashion.

|TRAVAUX

Ces travaux ont fait l'objet des productions scientifiques suivantes :

▪ PUBLICATIONS SCIENTIFIQUES

E. Boireau-Adamezyk, A. Baillet-Guffroy and G. N. Stamatas, "Age-dependent changes in stratum corneum barrier function", publication in **Skin Research and Technology** (SRT), 2014; 0: 1–7

E. Boireau-Adamezyk, A. Baillet-Guffroy and G. N. Stamatas, "Mobility of Water Molecules in the Stratum Corneum: Effects of Age and Chronic Exposure to the Environment**"**, **Letter to the Editor in Journal of Investigative Dermatology** (JID), 2014, 134: 2046–2049

E. Boireau-Adamezyk, A. Baillet-Guffroy and G. N. Stamatas, "Aging effects on the Physiology of the Stratum Corneum", Book chapter in **Skin Aging: Physiology, Clinical Aspects and Emerging Therapies**, 2015, Ed. Nova Publishers

E. Boireau-Adamezyk, A. Baillet-Guffroy and G. N. Stamatas, "Age-dependent changes in stratum corneum - Part II: water content and natural moisturization factors", submitted publication in **Skin Research and Technology** (SRT), 2015

E. Boireau-Adamezyk, Danielle Libong, Sana Tfaili, Georgios N Stamatas, Arlette Baillet-Guffroy, "Chronological aging impact on the skin barrier funtion: the Stratum Corneum lipid composition and molecular organization", submitted publication in **Journal of Lipid Research** (JLR), 2015

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E. Boireau-Adamezyk, A. Baillet-Guffroy and G. N. Stamatas, "Analysis of bound, weakly bound and bulk water inside the stratum corneum as a function of age and body site". ***Congress of International society for biophysics and imaging of the skin (ISBS)***, Milano, Italy, 2013

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E. Boireau-Adamezyk, A. Baillet-Guffroy and G. N. Stamatas, “Stratum Corneum Structure and Composition Evolve with age in Adults”. ***Congress of International society for biophysics and imaging of the skin (ISBS),***Copenhagen, Denmark, 2012

E. Boireau-Adamezyk, A. Baillet-Guffroy and G. N. Stamatas, “The Stratum Corneum Water Content and Biochemical Composition Evolve with Age and Depend on Body Site”. ***Congress of International Investigative Dermatology (IID),*** Edinburgh, UK, 2013

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1-O-E	1-O-Acyl ceramides
A	α-hydroxylated fatty acid chain
AA	Amino Acids
ACC	acetylCoAcarboxytase
Ala	Alanine
AR	Amphiréguline
ATR-FTIR	Horizontal Attenuated Total Reflectance – Fourier Transform Infrared spectroscopy
DRS	Diffuse Reflectance Spectroscopy
dS	dihydrosphingosine
dS-CER	Ceramide with a dS base
DTGS KBr	Deuterated triglycine sulfate detector, potassium bromide
ECM	Extra Cellular Matrice/ Matrice Extra Cellulaire
EO	ω-esterified fatty acid chain
FLG	Filaggrine
FT-MIR	Fourier Transform - Mid Infrared spectroscopy
FT-NIR	Fourier Transform - Near Infrared spectroscopy
Gly	Glycine
H	6-hydroxy-sphingosine
HA	Hyaluronic Acid
H-CER	Ceramide with a H base
HEX	Hexagonal packing
His	Histidine
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HR-MS	High Resolution - Mass Spectroscopy
HRNR	Hornerine
JED	Jonction Dermo-épidermique
L/P	Lipide/Protéine
LB	Corps lamellaire
LC	Long Chain
LC HR-MS	Liquid chromatography - High Resolution Mass Spectrometry
LIQ	Liquid phase
LPP	Long Period Periodicity
MMP	Matrix Metallo Proteinases
N	Non-Hydroxylated-/ non esterified- fatty acid chain

NMF	Natural Moisturizing Factors
NP-LC	Normal Phase Liquid Chromatography
O	ω-hydroxylated fatty acid moiety
OCT	Optical Coherence Tomography
Orn	Ornine
ORT	Orthorhombic packing
P	Phytosphingosine
PCA	Pyrrolidone Carboxylic Acid/ Acide Carboxylique Pyrrolidone
P-CER	Ceramide with a P base
PIE	Perte Insensible en Eau
Pro	Proline
PVA	Polyvinylalcohol
RH	Relative Humidity
RP-LC	Reverse Phase Liquid Chromatography
S	Sphingosine
SB	*Stratum Basale*
SC	*Stratum Corneum*
S-CER	Ceramide with a S base
Ser	Serine
SG	*Stratum Granlosum*
SPP	Short Period Periodicity
SPT	serine palmitoyl transferase
SS	*Stratum Spinosum*
SSLs	Skin Surface Lipids
T	dihydroxysphinganine
T-CER	Ceramide with a T base
TEWL	Trans Epidermal Water Loss
tsGAG	total sulfated Glycose Amino Glycans
tUca	Trans Urocanic Acid
UCA	Acide Urocanique
ULC	Ultra Long Chain
UV	Ultra Violet
VLC	Very Long Chain

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|INTRODUCTION GÉNÉRALE

La peau est un organe complexe d'une superficie d'environ 2m² et pèse environ 10% de la masse corporelle d'un être humain. Les tissus biologiques internes sont soumis a une dégénérescence irréversible plus ou moins rapide selon les facteurs physiologiques individuels tels que l'âge, le genre ou encore le statut hormonal. Ce vieillissement est appelé intrinsèque et des modifications cutanées observées chez les personnes âgées telles que l'apparition de rides accompagnent ce type de vieillissement. A cette sénescence biologique un autre type de vieillissement se superpose, généralement appelé vieillissement extrinsèque. La peau est soumise aux radiations solaires qui induisent une atrophie prématurée des couches superficielles de l'épiderme aux niveaux moléculaire et supramoléculaire. Ce vieillissement extrinsèque appelé également photo-vieillissement se traduit par des changements cutanés directement visibles ou non au niveau de l'épiderme comme les taches pigmentaires et plus en profondeur dans le derme comme la diminution du rapport collagène type I- collagène type III. Beaucoup de revues explicitent les changements dermiques mais peu reprennent les changements épidermiques aux niveaux moléculaire et supramoléculaire. Les évolutions démographiques de ces dernières décennies telles que l'allongement de l'espérance de vie et la diminution de la mortalité infantile ont conduit à une augmentation rapide de la population qui vieillit. Il est donc important de connaître les différents facteurs évoluant avec l'âge afin de pouvoir compenser les éventuelles modifications tissulaires, cellulaires, moléculaires et supramoléculaires.

Le *Stratum Corneum* appelé aussi couche cornée représente la couche la plus superficielle de la peau et est le siège de la fonction barrière. Cette membrane biologique très fine protège les tissus cutanés sous le *Stratum Corneum* de la dessiccation liée à un environnement aérien plus ou moins sec. Sans cette couche protectrice, l'espérance de vie serait très courte. Le *Stratum Corneum* joue donc un

rôle vital et fournit une protection en régulant les mouvements de va-et-vient de l'eau. Même s'il est déjà bien connu que le *Stratum Corneum* perd continuellement de l'eau à partir des couches les plus externes de la peau, la balance hydrique (différence nette entre la somme de la consommation d'eau et de la production d'eau endogène, moins la somme des pertes d'eau) ne doit pas être déséquilibrée afin de ne pas perturber l'homéostasie cutanée. Les mesures de la Perte Insensible en Eau, PIE, fournissent un paramètre généralement accepté pour la perméabilité de la fonction barrière et donnent l'information sur l'intégrité de la barrière.

Le *Stratum Corneum* joue également un rôle dans le maintien d'un niveau d'hydratation suffisant afin d'assurer une fonction physiologique cutanée efficace. Le maintien d'un état correct d'hydratation du *Stratum Corneum* à un impact important sur les propriétés mécaniques et optiques de celui-ci et est impliqué dans l'activation et la régulation des enzymes à la fois intra- et extra –cellulaires contrôlant le processus de desquamation. Il y a 60 ans, Blank et al. ont fait des observations intéressantes *in vitro* : des fragments plantaires isolés du *Stratum Corneum* deviennent durs et cassants lorsqu'ils sont déshydratés. Dans cette expérience *in vitro*, seule l'absorption de l'eau leur permet de redevenir souples et flexibles. L'eau peut être alors considérée comme la crème hydratante ultime qui améliore la perception subjective des propriétés mécaniques de la peau humaine. Cependant, même s'il y a toujours une source d'eau dans les tissus en profondeur *in vivo*, le *Stratum Corneum* de la peau sèche ne peut pas simplement capter les molécules d'eau de l'environnement pour garder la surface douce. Ainsi d'autres molécules hygroscopiques interviennent dans l'hydratation de la couche cornée et sont appelées facteurs naturels d'hydratation, NMF. Ces petites molécules présentes dans les cornéocytes sont des humectants très efficaces qui aident à retenir les molécules d'eau et jouent un rôle dans la flexibilité cutanée.

Au niveau moléculaire les cornéocytes qui sont riches en protéines du *Stratum Corneum* sont imbriqués dans une matrice lipidique contenant à la fois des céramides, du cholestérol et des acides gras. Cet empilement entre les cornéocytes et la matrice lipidique est souvent défini par un modèle dit « briques et mortier ». Les lipides de la matrice forment à la fois des couches lamellaires dans l'espace intracellulaire du *Stratum Corneum* mais ils ont également une organisation latérale donnant à la couche cornée des propriétés critiques aux niveaux mécanique et cohésif. A cause de la structure des céramides, de leurs propriétés et de leur poids (ils représentent environ 50% des lipides), beaucoup d'études portent sur cette classe lipidique.

Il est donc indispensable d'évaluer la distribution de la teneur en eau et des autres composés du *Stratum Corneum in vivo* tels que les NMF, les lipides ou encore les protéines afin d'expliquer l'évolution de la fonction barrière lipidique et de l'hydratation cutanée en vieillissant. Les méthodes électriques existantes estiment la qualité de la fonction barrière ainsi que l'hydratation de la couche cornée en un point précis de la surface. Cependant la peau et notamment la couche cornée comme d'autres tissus biologiques sont physiquement et chimiquement hétérogènes lorsque l'on regarde en profondeur et en surface selon le site corporel : la surface parait lisse à première vue mais à l'échelle microscopique elle est rugueuse ; au niveau tissulaire, l'épaisseur de la couche cornée n'est pas uniforme sur tout le corps humain ; ou bien au niveau cellulaire les cornéocytes s'aplatissent lors de leur migration vers la surface de la peau. Les concentrations chimiques des molécules dans le *Stratum Corneum* ne sont également pas identiques sur toute l'épaisseur de la couche cornée et varient de la surface vers l'intérieur : un gradient de concentration en eau existe du début de la couche cornée vers la surface. Les concentrations des constituants peuvent aussi varier selon le site corporel. Ainsi, il est nécessaire d'utiliser des méthodes performantes telles que des appareils d'imagerie avec une haute résolution digitale pour déterminer la composition et l'organisation de la structure au niveau moléculaire dans l'épiderme. Plusieurs méthodes non invasives et basées sur la spectroscopie vibrationnelle ont été développées ces derniers années afin d'évaluer de façon objective les propriétés

cutanées à la fois des peaux jeunes et âgées. Ces techniques non destructives ne nécessitent pas de préparation de l'échantillon ni d'utilisation de marqueurs particuliers. Elles peuvent être de plus utilisées *in vivo*. La spectroscopie infrarouge dotée du dispositif de Réflexion Totale Atténuée, ATR, est une technique permettant de déterminer la composition et l'organisation moléculaire à la surface cutanée. La micro-spectroscopie confocale Raman est une technique complémentaire permettant de déterminer les profils de concentration de certains composés par l'acquisition rapide de spectres et de déterminer la structure moléculaire du *Stratum Corneum*. Même si l'effet Raman est faible et que le risque de photo-vieillissement est limité, la détection nécessite tout de même une instrumentation sensible et très optimisée.

Dans ce travail de thèse nous avons étudié l'évolution de la fonction barrière *in vivo* avec le vieillissement intrinsèque et le vieillissement extrinsèque aux niveaux moléculaire et supramoléculaire dans l'épiderme et plus précisément dans la couche cornée.

Dans un premier temps nous avons répertorié les variations macroscopiques, chimiques, biochimiques, tissulaires, cellulaires, moléculaires et supramoléculaires décrites dans la littérature concernant le vieillissement intrinsèque et extrinsèque dans une revue bibliographique. Nous nous sommes concentrés sur l'épiderme et en particulier la couche cornée.

Dans une seconde partie, partie expérimentale, nous nous sommes intéressés à la fois aux changements de la fonction barrière et à l'explication de ces changements aux niveaux tissulaire, moléculaire et supramoléculaire induits par le vieillissement intrinsèque et extrinsèque pour des femmes caucasiennes séparées en quatre groupes d'âge différents au niveau épidermique et plus précisément dans le *Stratum Corneum* via des descripteurs Raman. Cette étude menée *in vivo* est la première étude réalisée en combinant à la fois des méthodes spectroscopiques vibrationnelles et des méthodes biométriques sur trois sites corporels simultanément, sur une population jeune (18 ans) à âgée (70 ans). Les trois sites corporels étudiés sont premièrement la joue qui a

un comportement différent du reste du corps comme par exemple le renouvellement cellulaire plus rapide ou l'épaisseur de la couche cornée plus mince. Le second site corporel étudié est le bras non exposé afin d'observer les effets du vieillissement chronologique. Enfin, le troisième site corporel étudié est le bras exposé au rayonnement Ultra Violet où les effets dûs au photo-vieillissement sont mis en lumière.

Suite à ce constat sur l'évolution de la fonction barrière avec le vieillissement, nous nous sommes intéressés à la matrice lipidique intercornéocytaire et plus particulièrement, au niveau moléculaire, les céramides qui jouent un rôle dans l'homéostasie cutanée. La chromatographie en phase liquide couplée à la spectrométrie de masse en haute résolution a permis de discriminer les différentes classes de céramides selon le type de population jeune ou âgée. Les variations de la fonction barrière au cours de l'âge ont justifié l'analyse poussée de la micro-hétérogéneité des céramides par une approche chimiométrique. Cette partie novatrice combine à la fois des méthodes chromatographiques et une méthode spectroscopique vibrationnelle : la spectroscopie infrarouge pour l'obtention d'informations en surface.

Puis dans une dernière partie, nous nous sommes intéressés à l'hydratation cutanée toujours sur les trois sites corporels précédemment explicités. L'hydratation dépend de deux paramètres : la teneur en eau dans la couche cornée ainsi que les facteurs naturels d'hydratation. Nous avons tout d'abord étudié plus spécifiquement la structure des molécules d'eau intervenant dans l'hydratation cutanée. Le rapport des différentes structures de l'eau varient selon la profondeur dans la couche cornée mais également selon le site corporel. Cette étude a été faite sur deux groupes de femmes caucasiennes d'âge différents. Les descripteurs Raman nous ont permis de caractériser les différents états de mobilité de l'eau. Puis, nous avons déterminé l'évolution des facteurs naturels d'hydratation via des descripteurs Raman pré-définis toujours sur les trois sites corporels précédemment explicités. Cette étude concerne toujours le vieillissement intrinsèque et extrinsèque *in vivo* pour des groupes d'âge différents.

ETAT DE L'ART

CHAPITRE:

1 VIEILLISSEMENT CUTANÉ

I INTRODUCTION AU VIEILLISSEMENT CUTANE

La peau est l'organe le plus lourd et le plus étendu de notre corps. La peau et plus particulièrement la couche cornée sont nécessaires à notre survie dans l'environnement afin d'empêcher notre desséchement. La peau possède une architecture complexe composée de plusieurs niveaux : épiderme, derme et hypoderme. Non seulement le vieillissement chronologique altère inéluctablement les fonctions physiologiques cutanées aux niveaux épidermique (Yaar et al. 2002) et dermique (Lavker 1979; Lavker and Kligman 1988; Gilchrest 1989) mais la peau est également soumise quotidiennement aux agressions extérieures (soleil et pollution). Les changements induits par le vieillissement intrinsèque et le photo-vieillissement (Gilchrest 1989) se caractérisent aux niveaux tissulaire (Black 1969; Shuster et al. 1975; Lavker 1979; Grove et al. 1982; Lavker et al. 1987) et cellulaire (Marks 1981).

1 | Histologie et Physiologie cutanée

|Organisation tissulaire

▪ L'ÉPIDERME

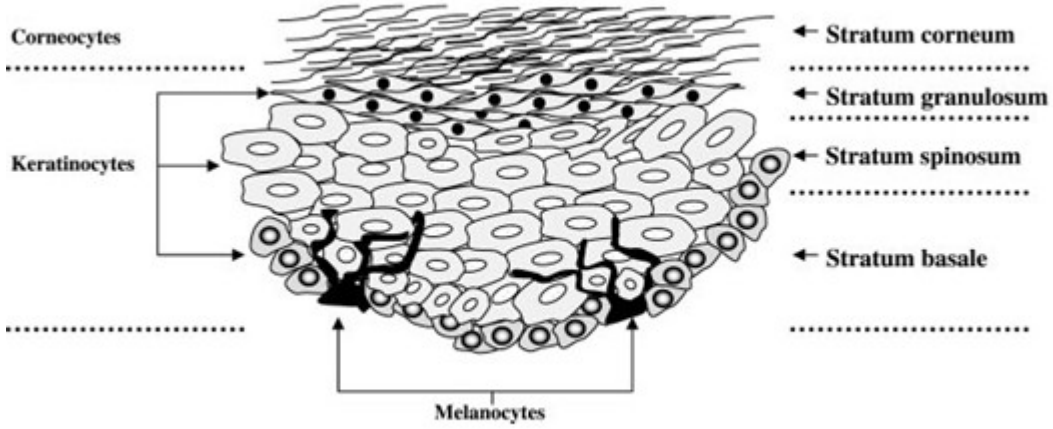
L'épiderme (**Figure 1**) est la couche la plus externe de la peau et mesure entre 5 à 100 µm d'épaisseur (Tobin 2006). Il contient principalement des kératinocytes (90% de l'épiderme), des mélanocytes et des cellules immunitaires appelées cellules de Langerhans (Jackson et al. 1993). L'épiderme est composé de quatre couches : le *Stratum Corneum* , SC, couche la plus superficielle, le *Stratum Granulosum*, SG, le *Stratum Spinosum*, SS, et enfin le *Stratum Basale*, SB, couche la plus profonde. Grâce à sa structure et son métabolisme, l'épiderme et notamment le SC protège la peau des

agressions extérieures et maintient un niveau d'hydratation suffisant dans les tissus internes (McCallion and Po 1993). Le SC dont l'épaisseur varie en fonction du site corporel contient environ 60% de protéines, 20% d'eau et 20% de lipides (Mathias and Maibach 1982; Jackson et al. 1993). Les kératinocytes sont produits dans la membrane basale à la Jonction Dermo-Épidermique, JED, et migrent vers le SC à la surface de la peau tout en se différenciant. Les kératinocytes lors de leur migration produisent les protéines cellulaires appelées kératine ainsi que les lipides. Les corps cellulaires s'aplatissent, perdent leur noyau et sont alors appelés cornéocyte (Tagami 2007). Les cornéocytes sont reliés entre eux par des desmosomes et sont couverts par une enveloppe hautement réticulée. Ils fournissent une barrière contre les agressions extérieures. Plus la fonction barrière est meilleure et plus la Perte Insensible en Eau est faible (Elias 2005). La barrière cutanée dépend également de la composition lipidique (Elias 1996) contenant du cholestérol, des céramides et des longues chaînes d'acides gras libres (Jackson et al. 1993; Elias 1996). Les lipides du SC sont hautement organisés et l'organisation à la fois lamellaire et orthogonale (**Figure 2**) contribue à la qualité de la fonction barrière.

La teneur en eau dans la partie viable de l'épiderme est maintenue à environ 70% (Caspers et al. 2003) et diminue à 20% lorsque l'on s'approche de la surface cutanée. La diminution s'observe à partir de la jonction entre le SG et le SC (Caspers et al. 2001). La capacité à retenir l'eau du SC (Tagami 2007) dépend des lipides, du sébum et des facteurs naturels d'hydratation appelés NMF, des acides organiques et des ions inorganiques. D'autres composés contribuent aussi au maintien de l'hydratation cutanée : l'acide hyaluronique (Sakai et al. 2000), le glycérol (Verdier-Sévrain and Bonté 2007) et l'aquaporin-3 (Sougrat et al. 2002). Le SG, ou couche granuleuse, est constitué de kératinocytes aplatis qui produisent des granulations de protéines appelées kératohyaline. Leur taille et leur nombre augmentent avec la dégénérescence du noyau cellulaire. Le SS, ou couche épineuse, est constitué de kératinocytes en forme de polyhèdre irrégulier et de cellules immunitaires appelées cellules de Langherans. Le SB, ou couche basale, appelé également *Stratum Germinatium* ou couche germinative, est

une monocouche attachée à la membrane Dermo-Épidermique qui sépare l'épiderme du derme. De plus, les kératinocytes de la couche basale sont situés dans cette couche et vont migrer avec des propriétés analogues aux cellules souches. Deux autres types de cellules sont trouvés dans cette couche basale : les cellules de Merkel ainsi que les mélanocytes.

a



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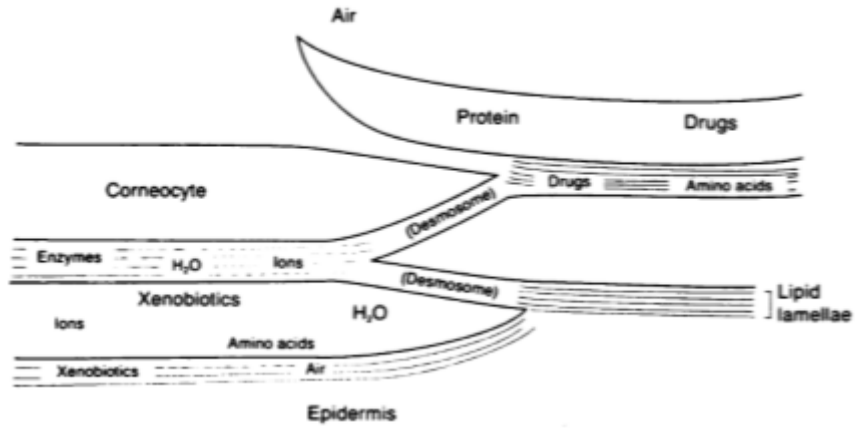

Figure 1 : Structure cutanée. *a) De H.Robins (1991) Biological Perspectives on Human Pigmentation, pp.2, Cambridge University Press, Cambrigde UK. Les couches de l'épiderme sont organisées du Stratum Basale, Stratum Spinosum, Stratum Granulosum et Stratum Corneum. La couche cornée est composée de cornéocytes, cellules mortes, intégrées dans une matrice lipidique, modèle appelé «brique et mortier »et b) De the Unilever Research, Colworth Laboratory, Sharnbrook, Bedford, England (DrJackson), et the Departments of Dermatology (Drs Williams and Elias) and Medicine (Dr Feingold), Department of Veterans Affairs Medical Center, San Francisco, and the University of California, San Francisco, School of Medicine. Les cornéocytes, appelés briques sont imbriqués dans une matrice contenant des lipides interconéocytaires, appelée ciment, organisés en couche lamellaires. Les espaces intercellulaires contiennent de l'eau, des ions et des enzymes résultant en une grande micro-hétérogénéité.*

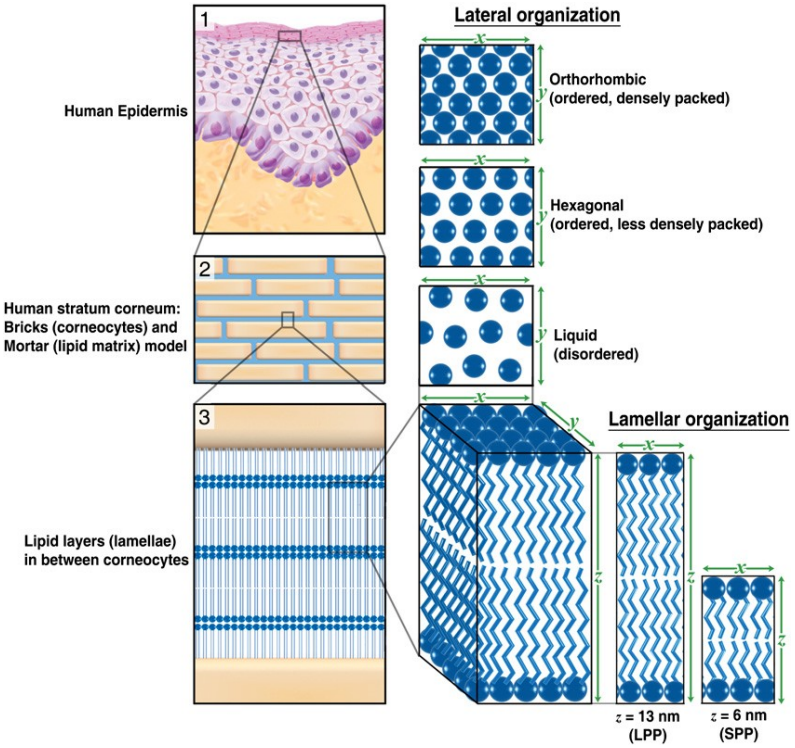

Figure 2 : Organisation lamellaire et latérale du SC humain. *De Janssens(M), Van Smeden J, Gooris G S et al. L'augmentation des céramides à courte chaîne corrèle avec une altération de l'organisation lipidique et une diminution de la fonction barrière chez les patients atteints d'eczéma atopique, J Lipid Res 2012: 53: 2755-2766. Les lipides intercellulaires sont disposés en couches (lamelles), avec deux phases lamellaires coexistantes. La distance entre la couche lamellaire appelée Short Period Periodicity, SPP, est de 6 nm, et de 13 nm pour la couche lamellaire appelée Long Period Periodicity, LPP. L'organisation latérale est contenue dans le plan perpendiculaire à l'organisation lamellaire. Trois dispositions sont possibles pour les lipides: une organisation orthorhombique ordonnée et très dense, une organisation hexagonale moins dense, et une organisation liquide désordonnée.*

JONCTION DERMO-ÉPIDERMIQUE

Entre les kératinocytes basaux et le derme papillaire, il existe une ligne ondulée permettant l'adhérence entre le derme et l'épiderme. La jonction dermo-épidermique est également appelée membrane basale et son ondulation est caractéristique des peaux jeunes. De l'épiderme vers le derme, elle est constituée de deux minces feuillets : la lame basale d'origine épithéliale et la lame réticulaire d'origine conjonctive. La lame basale est majoritairement constituée de collagène de type IV et de glycoprotéines. La lame réticulaire est constituée de collagène de type III. Les cellules épithéliales sont accrochées à la membrane basale, indispensable pour leur maintien et leur survie.

LE DERME

Le derme est un tissu conjonctif fibreux, élastique et beaucoup plus épais que l'épiderme, d'une épaisseur de 2 à 4 mm (Brincat et al. 2005). Il contient des molécules très organisées : l'élastine, le collagène et les fibroblastes qui ont différentes propriétés. Les fibres de collagène jouent un rôle dans la résistance cutanée face aux tractions (Brincat et al. 2005), l'élastine joue un rôle dans l'élasticité (Brincat et al. 2005) et les fibroblastes sont nécessaires pour la synthèse et la dégradation de la matrice extracellulaire, appelée ECM. Cette matrice extracellulaire est composée d'un mélange complexe de protéoglycanes, de glycoprotéines, de glycosaminoglycanes, d'eau et d'acide hyaluronique. Les structures dans le derme incluent trois annexes : a) les glandes sudoripares (eccrine et apocrine) et les glandes sébacées qui secrètent les triglycérides et le cholestérol ; b) les follicules pileux et les ongles et enfin c) les récepteurs nerveux sensoriels dits corpuscules de Merkel et de Meissner (pour le

touché), les corpuscules de Pacini (pour la pression) et les corpuscules de Ruffini (mécano-récepteurs).

- L'HYPODERME

Situé sous le derme, l'hypoderme est une couche de tissu conjonctif lâche contenant les plus grands vaisseaux sanguins de la peau et est constitué d'une couche de graisse pour le stockage d'énergie. L'hypoderme sert d'isolant et sert également à la thermorégulation corporelle.

2 | Composition cellulaire

L'épiderme est composé de différents types de cellules. Les kératinocytes sont présents dans l'épiderme et sont quantitativement majoritaires (80% des cellules épidermiques). Les kératinocytes produisent la kératine et migrent vers la surface cutanée par différenciation cellulaire. Les kératinocytes sont issus de la couche basale, forment une seule et unique couche cellulaire et sont reliés entre eux par des hémidesmosomes. Ils ont une forme cubique ou cylindrique. Ils migrent ensuite vers le SS qui est formé de 5 ou 6 couches de cellules selon la localisation. Lors de leur migration les kératinocytes s'aplatissent (Gilchrest 1996), jusqu'à devenir des couches de cellules très plates dans le SG. Les kératinocytes perdent leur noyau et sont alors appelés cornéocytes. Les cornéocytes forment la couche cornée. La différenciation cellulaire se termine par la desquamation des cellules afin de laisser place à de nouvelles cellules. Les mélanocytes produisent les pigments appelés mélanine qui colorent la peau. La mélanine joue un rôle dans la protection contre les UV. Les cellules de Langerhans sont des cellules mobiles dendritiques qui jouent un rôle

immunologique en contrôlant la présence d'antigènes. Enfin, les cellules de Merkel, également trouvées dans l'épiderme, sont des récepteurs du toucher.

3 | Vieillissement chronologique

a | Au niveau de l'épiderme

Le vieillissement cutané (**Figure 3**) résulte de combinaisons de plusieurs phénomènes : la diminution de la capacité de prolifération et l'altération cellulaire lors de la différenciation. Tous ces phénomènes conduisent à une modification de l'aspect cutané (Beauregard and Gilchrest 1987; Dewberry 2008); la peau s'atrophie, devient fragile et sèche. La couleur de la peau est de plus en plus pâle (Gilchrest 1982) jusqu'à devenir transparente laissant apparaître les composés dermiques, ceci est dû à la diminution de la rétro-diffusion. Le vieillissement chronologique a principalement une action sur le derme, qui perd sa fonction mécanique de soutien. Le renouvellement cellulaire des protéines est plus lent. La diminution de protéoglycanes et des glycoprotéines conduit à une perte de la tonicité de la peau. La synthèse d'élastine diminue avec l'âge, elle est alors remplacée par du collagène inextensible. Il en résulte des vergetures liées à des contraintes mécaniques. Le vieillissement chronologique s'accompagne de différents types de rides : les rides d'expression, les rides de gravité et les rides de surcharge. Les facteurs intrinsèques affectent la structure du visage et contribuent à la formation de rides d'expression sur le visage conduisant à de profonds sillons sur le front et entre les sourcils, péri-orbital et dans le pli naso-labial (Fitzpatrick et al. 1996). Ces sillons disparaissent lorsque la peau est étirée. La force de gravité (Donofrio 2000) joue également un rôle dans le changement d'expression du visage notamment, il en résulte un affaissement de la peau, particulièrement important sur les paupières supérieure et inférieure, sur les joues et dans la région du cou. Les rides

de surcharge sont liées à la modification du derme papillaire. Elles résultent d'un excès de synthèse de fibrocytes stimulé par les radiations ionisantes.

Lors du vieillissement, il y a également une perte des cheveux et une diminution de la thermorégulation qui conduisent à une incapacité à transpirer suffisamment pour diminuer la température corporelle et enfin le vieillissement s'accompagne d'une diminution sensorielle. Tous ces phénomènes s'accélèrent à la ménopause.

A partir de la soixantaine (Waller and Maibach 2005), la jonction dermo-épidermique s'aplanit d'environ 35% (Boss and Seegmiller 1981) à cause de l'aplatissement des papilles dermiques et elle perd ses ondulations (Boss and Seegmiller 1981). Il en résulte une diminution d'environ 70% de la surface de contact entre l'épiderme et le derme (Sans and Moraga 1993). Cette diminution augmente la fragilité de la peau et réduit les échanges de nutriments, d'oxygène et de métabolites entre le derme et l'épiderme (Südel et al. 2005). L'aplatissement limite également la prolifération des cellules basales et peut affecter l'absorption percutanée (Waller and Maibach 2005). Par conséquent l'épiderme est moins résistant à des forces de cisaillement et est plus vulnérable aux agressions extérieures en vieillissant (Hull and Warfel 1983; Grove 1989).

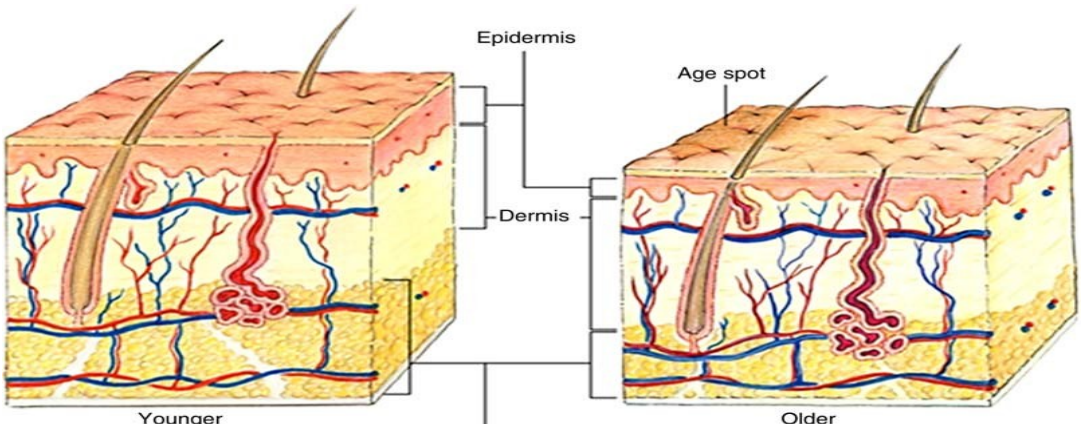

Figure 3 *: **Différences dans la structure cutanée entre population jeune et âgée**. KW Miller,P Elsner and HI Maibach. 2007. Structural Characteristics of the Aging Skin: A Review. Journal of Cutaneous and Ocular Toxicology 26: 343–357.*

b | **Au niveau cellulaire**

Globalement, le vieillissement chronologique s'accompagne d'une réduction du nombre de cellules dans l'épiderme. Cependant, les cellules souches épidermiques maintiennent leur niveau fonctionnel et sont résistantes face au processus de vieillissement, phénomène encore inexpliqué. La taille des cellules basales augmente en vieillissant (Brégégère et al. 2003).

Le nombre de kératinocytes diminue avec l'âge. La forme des kératinocytes devient irrégulière en vieillissant, ils sont plus courts et plus gros (Gilchrest 1996). Le vieillissement induit également des changements physiologiques rendant les kératinocytes insensibles a la stimulation (Yaar 1994). Ils perdent leur capacité de prolifération avec l'âge ainsi que celle d'élaborer de la cytokine. Une étude a montré que la diminution du 17β-estradiol progestérone avec la ménopause inhibe la prolifération des kératinocytes épidermiques (Tavakkol et al. 1999; Dieudonne et al. 2000). L'amphiréguline (AR)diminue également avec l'âge (Piepkorn et al. 1995). L'AR est un stimulateur autocrine de la prolifération des kératinocytes et de la synthèse des lipides.

L'intéraction anatomique entre kératinocytes et mélanocytes est appelée unité mélanocytaire épidermique et chaque mélanocyte est en contact avec environ 40 kératinocytes dans les couches basale et suprabasale (Fitzpatrick and Breathnach 1963). La taille des mélanocytes ainsi que celui de leurs dendrites varient également avec l'âge (Breathnach and Wyllie 1964; Gilchrest 1982). Des études ont également montré une diminution des mélanocytes de 8%-20% par décade après 30 ans, d'autres auteurs ont reporté une diminution de 10% par décade (Gilchrest 1996). Cette diminution aboutit aux taches de rousseur, lentigines et des naevus ainsi qu'à une diminution dans l'activité fonctionnelle des mélanocytes restants. Cette probable diminution peut provenir du fait que les mélanocytes deviennent indétectables lorsqu'ils ne produisent plus de pigments. Elle entraîne également une répartition

inégale de la mélanine dans la couche basale conduisant à une pigmentation inégale sur la peau des personnes âgées. La diminution des mélanocytes conduit non seulement à une couleur pâle aussi en partie attribuable à une diminution de la vascularisation mais également à un affaiblissement de la protection cutanée contre les rayons du soleil (Gilchrest et al. 1984).

Le nombre de cellules épidermiques de Langerhans diminue de 50%, entre 25 à 70 ans avec ~ 1200mm^{-2} pour les peaux jeunes à ~ 800 mm^{-2} pour les peaux âgées (Bhushan et al. 2002). Cette diminution induit une déficience au niveau de l'immunité cutanée. Elles deviennent plus hétérogènes avec l'âge (Wulf et al. 2004) et ont moins de dendrites (Grove and Kligman 1983; Wulf et al. 2004). Une étude a montré que la progestérone est capable d'augmenter le nombre de cellules épidermiques de Langerhans (Wieser et al. 2001).

Des études histologiques ont montré que l'aplatissement de la JDE résulte de la diminution du nombre de papilles dermiques par unité de surface passant de 40 papilles dermiques par mm² chez les peaux jeunes à 14 papilles dermiques par mm² chez les peaux âgées (plus de 65 ans).

4 | Photo- vieillissement

a | Au niveau tissulaire

Le terme de photovieillissement (Fenske and Lober 1986; Kligman and Kligman 1986; Gilchrest 1996; Benedetto 1998) a été utilisé pour la première fois en 1986 et décrit les effets des rayonnements du soleil sur la peau (Kang et al. 1997). L'exposition au soleil se divise en effets aigus et en effets chroniques. Les effets du rayonnement UV (UVA et UVB) sur la peau sont profonds et on estime qu'ils représentent jusqu'à 90% des signes visibles du vieillissement (Südel et al. 2005) en particulier chez les personnes sans protection naturelle c'est-à-dire des personnes ayant peu de mélanine qui pourrait les protéger de ces rayonnements (Robinson 1999). Les changements induits par

l'exposition chronique au soleil peuvent se produire bien avant l'expression du vieillissement chronologique.Le photovieillissement peut rester invisible pendant des décennies avant que les signes cliniques deviennent évidents. Les UVB affectent principalement l'épiderme, et sont directement absorbés par les cellules de l'ADN (dimères cyclobutane, pyrimidine (Allsopp et al. 1992; Phillips et al. 2001), et photoproduits pyrimidiniques). La peau devient hyperplasique sous l'effet de l'exposition au soleil (Glogau 1997). Cette augmentation de volume résulte de l'exposition aux rayonnements solaires. La peau soumise aux rayons UV perd également son élasticité, devient rugueuse et sèche (Yaar et al. 2002). Les rides sont beaucoup plus profondes (Kligman and Kligman 1986) et marquées que les rides induites par le vieillissement chronologique. Les changements pigmentaires sont beaucoup plus nombreux (Makrantonaki and Zouboulis 2007): apparition de taches dites « taches du soleil ». Il peut y avoir également une dépigmentation ou encore une pigmentation irrégulière comme l'apparition de la dyschromie (Glogau 1997).

b | **Au niveau cellulaire**

Les radiations solaires conduisent à des changements dans le nombre de cellules et dans leur fonctionnalité. Ces changements peuvent commencer dès l'enfance. Les longueurs d'onde les plus courtes de la lumière UV (UVB) sont absorbées dans l'épiderme et affectent les kératinocytes épidermiques (Garmyn et al. 1992). Les kératinocytes épidermiques sont la principale source cellulaire de MMP (Varani et al. 2000; Chung et al. 2001) produits en réponse à l'exposition de la peau humaine au rayonnement UV. Les radiations solaires conduisent également à une désorganisation et une atypie cytologique des kératinocytes (Kligman and Kligman 1986; Gilchrest 1996). Le rayonnement UVB conduit à une diminution de la croissance des kératinocytes et augmente leur différenciation (Pâquet et al. 1996), le rayonnement UV active aussi l'expression des récepteurs de facteurs de croissance des kératinocytes (Fisher et al. 1998). L'une des premières réponses des kératinocytes à l'irradiation UV est la libération de cytokines pro-inflammatoires d'une manière similaire à la réponse

due aux blessures. Suivant l'irradiation aigüe ou chronique aux UV de la peau humaine *in vivo* (Seo et al. 2001), ou des kératinocytes humains en culture (Seo et al. 2001), les kératinocytes contiennent une plus grande quantité de tropoélastine ARNm. Il a été montré que les niveaux d'ARNm tropoélastine sont plus grands sur l'avant-bras face postérieure (partie exposée au soleil) de la peau des personnes âgées par rapport à la partie du bras face antérieure (partie non exposée au soleil) de la peau des mêmes individus (Seo et al. 2001).

Avec le photovieillissement, les cornéocytes deviennent polymorphiques avec des anomalies croissantes : conservation de restes du noyau et rugosité des bordures du cornéocyte (Grove 1989; Bergfeld 1996).

Des différences pigmentaires existent entre les effets de l'exposition au soleil aiguë et l'exposition chronique. L'exposition aigüe induit une pigmentation immédiate et retarde la formation de nouvelle mélanine. Cette réaction est réversible. Au contraire, l'exposition répétée au soleil conduit également à un disfonctionnement dans l'homéostasie des mélanocytes : augmentation du nombre de mélanocytes, stimulation dans la synthèse de la mélanine, augmentation de la dendricité mélanocytaire, caractéristique morphologique essentielle requise pour le transfert de mélanine aux kératinocytes. Le rayonnement UV entraîne alors la pigmentation générale de la surface de la peau avec une pigmentation cutanée irrégulière. La distribution de mélanine est alors irrégulière dans la couche basale épidermique. Une étude menée par Lavker et al. (Lavker et al. 1995) suggèrent que si les UVA sont administrés au cours du temps, ils peuvent induire des changements similaires à ceux induits par les UVB, y compris la diminution des cellules de Langerhans.

CONCLUSION

La peau est un organe sensoriel que l'on pourrait décrire comme une usine de synthèse. Les données bibliographiques concernent principalement la physiologie cutanée, peu décrivent l'évolution du SC avec le vieillissement aux niveaux moléculaire et supramoléculaire. L'objectif de ma thèse porte plus précisément sur l'étude du vieillissement cutané *in vivo* par des méthodes non invasives au niveau de la couche cornée. La fonction barrière maintient le niveau d'hydratation cutanée au cours du temps. Ainsi, le paragraphe suivant s'oriente sur l'évolution de l'hydratation cutanée et la fonction barrière au cours du vieillissement chronologique et le photo-vieillissement. Les modifications de la composition moléculaire et de l'organisation supramoléculaire de la couche cornée seront mises en lumière.

II CHAPITRE DE LIVRE :VIEILLISSEMENT CUTANÉ ET PHYSIOLOGIE DU SC

Chapter

AGING EFFECTS ON THE PHYSIOLOGY OF THE STRATUM CORNEUM

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Abstract

The advent of non-invasive methods has enabled the study of skin physiology *in vivo*. A wealth of information can thus be acquired about the effects of intrinsic and extrinsic aging, also referred to as chronological aging and photoaging correspondingly, on skin structure, function, and composition. While a lot of work so far has focused on the effects of aging on skin appearance, few studies have dealt with the aging-related changes of the skin barrier and more specifically on the physiology of the epidermal layer that provides most of the barrier function, i.e. the Stratum Corneum (SC). The characteristic structure and composition of this upper part of the human skin confers a veritable barrier to the penetration of external irritants as well as excessive loss of internal water which could lead to dehydration. In the present chapter we will review the relevant methods, the physiological structure and function of the SC, as well as the effects of intrinsic and extrinsic aging on these parameters.

Introduction

The skin is a sensory organ and a biofactory for the synthesis, processing, and metabolism of a wide range of structural proteins, glycans, and lipids [1] fulfilling all the requirements of a classic endocrine organ. [2,3] The role of the skin is to protect the organism from water loss, temperature change, radiation, and trauma. The important role of the barrier function resides primarily on the upper-most layer of the epidermis, the SC. Non-invasive methods involving probes that can come in contact with the SC can be used to determine the physiological structure and function of the SC *in vivo*. Aging is a progressive process involving reduction in optimal function and renewal capacity of the whole organism. [4] It results in cellular attrition and senescence, eventually terminating by decreased viability and death. It is orchestrated

by a genetic program but is also affected by cumulative environmental and endogenous insults that take place throughout the organism's lifespan. The causes of chronological aging are less clear than those of the second type of aging, so called extrinsic aging or photoaging. Photoaged skin is the result of exposure to environmental elements in particular UV radiation, superimposed to chronological skin aging. It sometimes leads to prematurely aged appearance even in young people and is linked to immune suppression. Most of facial aging can be attributed to sun exposure. [5] While some sun exposure is necessary for the production of vitamin D, excessive exposure can lead to tissue damage. Compared to the dermis, little is known about the effects of aging on the epidermis and more specifically on the SC.

The Structure of the Epidermis

The epidermis is the outer, non-vascularized, stratified epithelium, that measures 5 to 100 µm in thickness (although it can reach 600 µm or more on the palms and soles). [6] It is composed of several distinct cell populations, primarily keratinocytes (90% of the epidermis), melanocytes, and immune cells (Langerhan's cells). [7] The epidermis is arranged in four layers: underneath the SC one finds sequentially the Stratum Granulosum (SG), the Stratum Spinosum (SS), and the Stratum Basale (SB). In the palms and the soles there is also the Stratum Lucidum, which consists of flattened cells with no nuclei. The epidermis fulfills two main functions due to its structure and metabolism: it protects the skin from external insults and it maintains the hydration of internal tissues. [8] Both functions are realized primarily by the SC, the outer most layer of the epidermis. [9]

The keratinocytes originate from dividing cells at the SB, which is in contact with the basement membrane defining the Dermal-Epidermal Junction (DEJ). These cells

continue to differentiate as they move from the basal layer to the SC. As the keratinocytes move toward the skin surface, they produce the definitive skin cell protein, keratin, as well as lipids. Their shape changes as they mature. The flattened cell bodies of dead keratinocytes are called corneocytes and they are covered by a highly cross-linked and cornified envelope. [10] The corneocytes provide the barrier against environmental insults. The strength of the epidermal barrier function is inversely related to the rate of Trans-Epidermal Water Loss (TEWL). [11] The SC contains about 60% proteins, 20% water, and 20% lipids. [7,12] The efficacy of the water barrier also depends on the SC lipid composition [9] is comprised of cholesterol, ceramides, and long-chain free fatty acids. [7,9] Both lamellar and lateral lipid organizations contribute to the quality of the barrier function.

Thanks to the SC structure and composition, the water content of the viable portion of the epidermis is maintained at about 70% [13] and decreases toward the skin surface to about 20% w/w. This decrease begins to be observable at the juncture between the SG and the SC. [13] Intercellular lipids, as well as the Natural Moisturizing Factors (NMF), organic acids, and inorganic ions, impact the water-holding capacity of the SC. [10] Several less abundant components in the SC also contribute to maintaining skin hydration, such as Hyaluronic Acid (HA) [14] and glycerol. [15] Moreover, below the SC, aquaporin-3 at the surface of keratinocytes contribute to the water mass balance in the skin. [16]

The SG contains flattened, polyhedral non-dividing keratinocytes producing granules of a protein called keratinohyalin. The size and the number of these granules increase as the cell nuclei gradually degenerate leading to cell death. These cells flatten as dividing cells underneath them progressively push them toward the skin surface.

The SS contains irregular polyhedral keratinocytes and Langerhan’s cells. The Langerhan’s cells are bone marrow-derived sentinel cells of the immune system. They function as antigen-presenting cells of the skin playing a role in immunological reactions such as allergic contact dermatitis. In the SS the cell division capacity is

limited.

The SB is also known as the Stratum Germinatum. It is a single layer of cells attached to the basement membrane at the DEJ that separates the epidermis from the dermis. Moreover the basal keratinocytes are situated in the SB and will migrate toward the skin surface. Two other types of cells are found within this layer: Merkel cells (neuroendocrine cells responsible for the transmission of touch sensation through the cutaneous nerves) and melanocytes.

Non-Invasive Methods

Age related changes can be measured by different methods: electric, spectroscopic, chromatographic methods, and imaging applied on biopsy samples or *in vivo*. As it is best to study the tissue in its native state, we will focus on non-invasive methods that are adapted to be used *in vivo*.

Electric-Based Methods

These methods indirectly assess the SC water content by measuring the skin electrical proprieties (impedance, capacitance, and conductance) that depend on the amount of water in the SC. The Corneometer for example measures the ability of the skin to store charges as an electrical capacitor, a property called cutaneous capacitance. [17] The value varies as a function of the water content inside the SC: the more hydrated the SC is, the easier the electric current can flow through the top layers of the epidermis due to the higher ionic mobility in an aqueous environment. Although they are very easy to use, these electric methods have some limitations partially due to the skin lipids. Lipids can prevent the electric flow to pass through, decreasing the skin conductance values

and increasing the measured skin capacitance. The concentration of total ionic species may also interfere with the electric conductivity of the skin.

The quality of the SC barrier function can be assessed by the measurement of the rate of TEWL through the SC. When the body is at rest, there is a constant value of in-and-out movement of water resulting in a mass balance equilibrium. Another way to measure the water barrier function is to measure the skin moisture content during a sorption/desorption test. [18]

Spectroscopic Methods

All *in vivo* spectroscopic methods rely on the interactions of light with the components of the tissue under investigation.

Diffuse reflectance spectroscopy (DRS) [19] is used to determine the apparent concentration of absorbing molecular species in the UV wavelength called chromophores. In the skin these include melanin, oxygenated hemoglobin, and deoxygenated hemoglobin. The spectra obtained from the tissues have spectral characteristics of these chromophores. [20]

Fourier Transform–Near Infrared (FT-NIR) and Mid Infrared (FT-MIR) [21] spectroscopies are based on energy absorption due to molecular bond vibrations. The frequency at which energy is absorbed is characteristic of one kind of the chemical bonds in the structure being analyzed. For example methyl, ethyl, carboxyl groups in their microenvironment can be studied. However, energy absorption of water molecules in the NIR range is very strong and it is difficult to obtain information about other molecular species. It is therefore beneficial to limit the sampling volume to the most superficial layers of the SC that contains about 20% of water. To sample only the superficial layers of the skin it is necessary to use a dispositive called Attenuated Total Reflectance (ATR) crystal. [20] Using this type of probe only the very superficial part of

the skin is studied (1 to 2 µm depending on the wavelength). ATR-FTIR spectra are obtained providing detailed vibrational information on molecular composition and molecular structure. Good and reliable measurements of water and skin lipids are obtained, whether they come from sebaceous glands or the SC. Surface information about the lipid organization with the FTIR technique can be obtained. Tissue absorption rates of an active compound [22] can also be studied by the rate of reduction of its surface concentration.

Raman spectroscopy [23] is another vibrational method that is a well-adapted tool for studying *in vivo* the supramolecular organization and biochemical composition of the SC. *In vivo* confocal Raman spectroscopy is able to measure spectra at specific depths from the skin surface down to 100–200 µm. [24,25] The penetration depth is limited by multiple scattering events, the numerical aperture of the objective, and the photon absorption in the tissue. Structural and organizational information of molecular bonds have characteristic signatures in Raman spectroscopy. It is possible to deduce the nature and the structure of a molecule in free or bonded forms and also the interaction with its environment. [26] This method can be used to collect information about the quality of the SC barrier function, the hydration state of the SC, and the SC thickness. It can also be used to study the penetration of an active substance and its concentration distribution through the SC.

Fluorescence spectroscopy has been used to study skin physiology. [27–39] It has been shown that dry keratins fluoresce brighter than when hydrated. The epidermal cell proliferation rate has been related to the signal of tryptophan moieties (excitation maximum at 295 nm and emission maximum at 345 nm). [30] It has been shown that when the rate of the epidermal cell proliferation increases, the tryptophan signal also increases. [27] Biochemical stimulation [29] or exposure to UV radiation [27] may induce tryptophan fluorescence. The tryptophan fluorescence intensity decreases with aging reflecting the decreased capacity of epidermal cells to proliferate.

Imaging Methods

Ordinary visible light microscopy can be used to get information about the micro relief line density and patterns, pore size, and melanin distribution.

The spectroscopic adaptation of imaging (e.g. fluorescence or spectral imaging) can be used to examine the spatial distribution and variation of molecular concentrations of interest. This is an extension of the single point information that it is otherwise obtained by regular spectroscopy.

Spectral imaging can be used to identify and quantify chromophores or molecular species due to their characteristic absorption bands. [40,41] The skin depth probed depends on the absorption and scattering proprieties of the skin and on the wavelength of the light source.

The chemical information obtained by Raman spectroscopy can be combined with the spatial distribution in Raman microscopy. Raman spectral imaging at the cellular level has been used to get information relating to apoptosis, cell growth and differentiation, and product-cell interaction. [42]

In vivo Reflectance Confocal Microscopy (RCM) is used to capture light that has gone through a single scattering event in the tissue. [43] The amount of collected light is limited by the numerical aperture of the objective and photon absorption. The laser source is scanned in a horizontal plane that optically transverses the tissue. At a cellular level architectural details that can be evaluated including epidermal thickness [44,45], keratinocyte disorder with aging [46,47], the compaction of keratin in the SC, the effects of aging on melanocytes, the distribution of melanosomes inside melanocytes, melanin production, and changes in the nuclei.

Optical Coherence Tomography (OCT) [43–45,48–53] is a non-invasive imaging method generating vertical cross-sectional images of the skin capable to present morphological features of the epidermal and papillary dermis. The axial resolution (about 5 μm) and

the quality of image are comparable to RCM. The recorded signal depends on the optical proprieties of the skin and primarily scattering. This method involves a fiber optic Michelson interferometer with a low coherence length broadband light source as IR. [53,54] OCT is not capable to routinely differentiate between the SC and the viable epidermis. The determination of the mean skin thickness is in agreement with cryostat-histology, considered as the gold standard to assess the epidermal thickness. [55]

The phenomenon of two-photon microscopy [56] occurs when two photons with the same wavelength interact and their energy recombines resulting in a single photon that has double their frequency (or equivalently half their wavelength). A pulsed laser is used with a high repetition rate (MHz range) and with very short pulses: 10^{-15}s. The laser light (typically in NIR) excites the UV fluorescing molecular species in the skin. It is able to propagate deep into the skin as the absorption and the scattering coefficients are minimal.

Chromatographic Methods

Chromatographic methods, including gas and liquid chromatography are able to provide molecular information for distinct molecular groups, such as lipid species to result in lipid profiles. [57] In general, liquid chromatography (normal phase or reverse phase) or gas chromatography are used with a detection system, which can be the evaporative light scattering detector or charged aerosol detector (Corona) [58] for quantitative studies or High Resolution Mass Spectroscopy (HR-MS^n) for qualitative studies. Skin surface lipids (also named hydrolipidic film) and SC lipids can be acquired with minimally invasive methods such as absorbing papers for the first type and tape-stripping or solvent extraction for the second. These analytical techniques are particularly useful for generating lipid profiles [59] because of the complexity of the hydrolipidic film lipid or SC lipid composition. The importance of ceramides sub-classes and their length and unsaturated chains on compactness of lipid edifices has been

clearly shown. Only the chromatographic methods can give qualitative and quantitative information about these compositions. [60,61] This kind of molecular information is very interesting because it can give some explanation of the aging effects on the barrier properties.

Skin Aging

Intrinsic (Chronological) Aging

Visual Appearance [62,63]

The processes involved in wrinkle formation have been discussed by others. [64,65] Here we will focus on aging effects on the epidermis and more specifically its outer most part, the SC. Skin atrophy is accompanied by an increase in fragility, dryness with pruritus and xerosis with the apparition of a variety of benign neoplasms such as seborrheic keratoses and angiomas. With age the skin becomes more susceptible to irritation [66] and there is a decrease in sensorial perception.

Thickness of the SC and the Viable Epidermis

Some authors found that the total epidermal thickness remains constant with increasing age. [67,68]

Concerning the thickness of the SC histological studies show different results: some observed that the SC thickness does not change with age [69,70] and some authors

found a slight increase. [71]

Some studies report that thickness of the viable epidermis tends to decrease [72–74] progressively at a rate that accelerates with age. [75] Indeed, the overall human non-exposed epidermis decreases by ~10-50% between 30 and 80 years old [70,76] equivalent to 6% per decade. [77,78]

Not only the thickness of the total epidermis decreases but also the resistance to pressure is diminished. [74,79] These different conclusions can be explained by the small physiological variations and depend on the group of age of interest.

Barrier Function

The skin barrier function is complex. It is primarily located in the SC and is the result of many factors including the composition and the organization of the intercellular lipid cement. The strength of the barrier function depends on the location of the body site of interest. [71] The results published in the literature are sometimes contradictory as the employed method, the population demographics, and/or the skin site may be different.

The most common method to evaluate the quality of the skin barrier function is by measuring the TEWL.

In the published literature one finds different results concerning the evolution of the barrier function with aging: some studies demonstrated no change in TEWL rates with aging [80,81] indicating that the barrier function is not altered significantly with aging. [81–83] Other studies show that the barrier function slightly improves with aging. [71;10,84–86] However, the causes of reduced rate of TEWL of aged epidermis are not known. [80]

The barrier function can also be evaluated by the measurement of the penetration rate

in the epidermis of exogenous molecules topically applied. In onereport caffeine penetration has been proposed as a marker to evaluate the perturbation of the barrier function. [87]

Research has been recently focused on the origin of the barrier function at the molecular and supramolecular level. Chromatographic and spectroscopic methods are used to study the age-dependent evolution of the hydrolipidic film and the SC lipid composition and organization.

Although some studies indicate little or no relationship between age and lipid content [88,89], many authors accept that overall total lipid content decreases in the SC with age [71,90] by about 30%. This reflects the slower keratinocyte metabolism of the aged skin [91,92] that results in a decrease in biosynthetic capacity. However, Cua et al., [88] compared lipid composition with small angle X-ray diffraction and they did not find differences between an older and a younger group: relative ratios of each of the major lipid classes (cholesterol, fatty acid and individual ceramide species) are constant throughout life.

Denda et al., [93] showed by High Performance Thin Layer Chromatography (HPTLC) that from puberty to adulthood there is an increase of the ratio of the ceramide1 (or EOS) and ceramide 2 (or NS) to the total sphingolipid amount and a decrease of the ratio ceramide 3 (or NP) and ceramide 6 (or NH) to the total sphingolipid amount (definition of subclasses according to the classification by Rabionet et al., [94]). The ratio of ceramide 2 to the total sphingolipid amount decreases with age and that of ceramide 3 to total sphingolipid increases with age. Moreover, the SC sphingolipid composition depends on the epidermal biosynthesis of sphingolipids that is influenced first by the epidermal proliferative activity [95] and secondly by the presence of hormones. This also suggests that the age-associated decrease in hormone levels may impact the skin barrier function in the elderly. [93]

Rogers et al., [91] found by HPTLC that all classes of ceramides decrease by 30% with

ageon the face, hands, and legs in older subjects (limited to subjects up to 50 years of age). It is most marked for the levels of ceramide species 1-6, on the face and the hand but the percentage ratios of the individual species remained constant. The SC lipid levels also depend on the skin site (face, hands and legs). Ceramide 1 linoleate decreases with age on the leg. This depletion of ceramide 1 linoleate contributes to the formation of an intrinsically weaker SC with an increase of susceptibility to dysfunction of desquamation and xerosis. Age-effects on SC lipids are not limited to ceramides. Rogers et al., [91] and separately our team [71] found that cholesterol decreases on the face, although the percentage ratio of cholesterol to lipid is constant. They also found a decrease in fatty acid levels, but with a constant ratio of fatty acid to lipids. [91,92,96] Makrantonaki & Zouboulis showed that the cholesterol content decreases [97] on the cheek with age. Saint Léger et al., found a decrease in sterol esters and triglycerides with age [92] on the lower legs.

The decline in cholesterol, ceramides and free fatty acids content can be attributed to the reduced activities of key rate-limiting enzymes for each of these lipids including serine palmitoyltransferase (SPT), 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, and acetyl CoA carboxytase (ACC). [98]

Lipid organization plays a key role in the barrier function. [99–101] Lipids form multilamellar sheets within the intercellular spaces of the SC, the organization of which is essential in maintaining the functionality of the skin as an effective barrier to water loss. [102,103] Spectroscopic methods are pertinent tools for this purpose. The conformational order is reported to be directly related to the lateral packing. [104–106] Tfayli et al., showed that aging leads to the loosening of the organizational order and a decrease in the ordered inter-molecular structure. [107] In parallel they also showed that with aging there is a decrease of the trans/gauche conformation structures in the alkyl chain of lipids indicating a lower intra-chain order and inducing an increase in the lateral packing disorder. [107] Our team has reported a decrease of the lipid compactness on the exposed arm site and on the protected arm site with

aging. [71]

Interestingly, Cua et al., [88] and Saint Léger et al., [92] found no relationship between the skin surface lipid content and age except on the ankle, with the older group showing an increase in lipid content. Other authors report that the skin surface lipids at a very old age are similar to those of pre-pubertal children. [108] However, sebum production could be a more complicated process between the young people and the elderly. Sebaceous glands are well developed in neonates but their size tends to change with age. [109] They become smaller a few weeks after birth and they are almost undetectable during childhood. [110,111] Their size increases again with the adrenarche, develops further with the onset of puberty, reaches its maximum at the 3rd decade of life, remains constant until after menaupose, tends to decrease slowly at the 7th decade [112], and shows no significant change after the 7th decade. As for the keratinocytes, 17 β-estradiol progesterone (during menaupose) reduces sebaceous differentiation. [113,114] The concomitant decrease in the production of androgens may explain the decrease in sebaceous gland synthesis and secretion. Moreover the turnover rate of the sebaceous glands in older skin is reduced compared to young. Other factors such as hormones, gender, and race influence the sebum secretion besides age. Sebum production on the human face has been found to vary with age according to Le Fur et al., [108] However, there is a paradox concerning the aging of facial skin. On the face the volume of the sebaceous glands increases with age but paradoxically their secretory output decreases. [109,111,115] accompanied by a decrease in the surface lipid levels. Although sebum production declines in old age, the size of the follicular reservoir may not because the sebaceous glands undergo hypertrophy in older individuals. [115]

Other factors implied in the barrier function should be considered such as the pH and the calcium gradient.

Skin surface pH is relatively constant from childhood to approximately 70 years of age [77] then rises significantly. This phenomenon is especially pronounced in lower limbs

[77] possibly due to impaired circulation. [77] Wilhelm et al., and Zlotogorski also showed that facial skin pH is more alkaline in aged subjects and that regional differences exists among the various areas of the face. [83,116] Wilhelm et al., found that the forearm has the most alkaline pH and the forehead has the most acidic pH that increases with aging. [83]

The role of calcium in lamellar body secretion is well known. Altered calcium gradient in aged epidermis could account for the barrier abnormality. [117] Aging blunts the calcium concentration gradient through the epidermis [117] due to a decrease in the activity of ions pumps, ions channels, and/or ionotropic receptors in aged skin. In aged human epidermis both the internal structure of lamellar bodies, as well as their numbers in the cytosol of granular cells, are similar to those in young subjects. However, variations were evident in the structures of the unsaturated fatty acids. Aged epidermis displays a decrease in secreted lamellar body-derived contents and a failure of these contents to form a continuous series of multilamellar bilayers within the intercellular spaces of SC. [80]

Following acute insults, barrier recovery is delayed in aged skin. [80,98,118] Functionally the time required to recover from an epidermal wound, for example blistering, increases by about 50% between youngand older adults. [95,119] It takes 3 days in young adults and 6 days in elderly adults to recover from a blistering insult. [80] In young epidermis, barrier abrogation is followed by a repair process that involves: 1) immediate secretion of preformed epidermal lamellar bodies; 2) replenishment of the cytosol with newly formed lamellar bodies and further secretion of lamellar body contents; 3) formation of lamellar bilayers in the SC interstices leading to recovery of the barrier to TEWL within 24 hours.

The aged epidermal permeability barrier is easier to perturb. Older skin seems to absorb topical substances more slowly than young skin. [120] Depending on the compound there may be differences in the rate of percutaneous absorption, which may explain conflicting results concerning the percutaneous absorption. [121] The rate

of percutaneous absorption also depends on the body site. [90] Hydrophobic compounds penetrate more easily in areas of the body skin that have a high percentage of skin lipids. [90]

SC Water Content

Concerning the effects of aging on the SC water content conflicting reports exist in the literature, either showing that SC water content does not change [83,122,85] or that it slightly decreases. [7,8,71,75,124] The vibrational energy of OH bonds in the water molecule depends on the formation of hydrogen bonds at the local microenvironment. Water molecules that form non-covalent bonds to other compounds have been termed as called “bound” water. Other water molecules form hydrogen bonds with neighboring water molecules in a tetrahedral structure. These molecules are called tetrahedron or bulk water. [124–128]

Intrinsic aging does not appear to alter water structure significantly. [129] Gniadecka et al., show that in the epidermis of young skin the water is present mainly in “bound” form. [130,126,127,131] Vyumvuhore et al., distinguished between bound water, weakly bound, and bound water in the SC *ex vivo* using confocal Raman microspectroscopoy. [132] Following that report our group showed in an *in vivo* study that the distribution of the three states of water according to molecular mobility are independent of age. [133] Bulgin & Vinson [134] showed by differential thermal analysis that the bound water represents 34% of the normal SC dry weight that is equal to 80% of the total SC water. The water-holding capacity of the SC depends on the skin site. Egawa & Tagami showed that the SC water content at a depth of 10-30 µm in the forearm skin tends to be lower in older subjects than in younger subjects. [135] On the other hand no difference was found in the much thinner SC of the cheek between different age groups in facial water-holding capacity and skin conductance.

Mechanical Function

The SC water content plays an important role in controlling the activity of the enzymes involved in the corneocyte desquamation. Reduction in the levels of SC water content can lead to altered desquamation and accumulation of corneocytes at the skin surface, resulting in skin surface roughness, scaliness, and flaking that accompany xerosis in aged skin. The corneocytes become larger due to a decreased epidermal turnover rate. [121,136] They clump together on the surface, the surface becomes rough with a scaly appearance and texture. [137] The increase in corneocytes size that accompanies aging process [138] may compensate for the effects of reduced lipid content.

Vyumvuhore et al., studied the behaviors of the SC components during water desorption, following a drying process using Raman spectroscopy. [139] Mechanical stress of the SC mainly depends on the concentration of the bulk water.

Epidermal Cell Turnover Rate

Keratinocytes generated in the basal layer migrate toward the SC, where they become corneocytes and finally are eliminated at the skin surface. This epidermal cell turnover rate depends on the skin body site and the age.

Epidermal cell turnover rates decrease by about 50% from 20 to 70 years of age, which leads to epidermal atrophy at the histological level and to a thinner epidermis. Even if there is a decrease of the epidermal cell proliferation the number of cells layers remains stable. [121] TGF-β1 could be a potent inhibitor of epidermal growth as this multifunctional cytokine regulates cell proliferation and differentiation, tissue remodeling, and repair. In young and healthy skin, one layer of corneocytes desquamates every day. The whole SC replaces itself in about 15-20 days [10] in young skin in contrast with the elderly SC that may take twice as long, 40-60 days. [95,140]

The decrease in epidermal cell turnover rate may in part explain the decrease in skin barrier function and why the repair process is delayed in the aged. [120]

Globally, there is an overall reduction in epidermal cell number [141] with aging in the epidermis. Senescent cells are accumulating in aging human skin. Decline in the integrity and function of the skin and possibly other tissues, associated with aging, is due it the presence of senescent cells.

Biochemical Composition

Skin aging is accompanied by a decline in the NMF production according to McCallion. [8] The term NMF represents a mix of hygroscopic molecules: free fatty acids, Pyrrolidone Carboxylic Acid (PCA), urea, lactate ions, and sugars. The NMF helps retain water molecules in the SC. In older people the SC of the cheek and the forearm contains more NMF than the SC of young individuals. The increase of the NMF content may be the result of a slower SC turnover rate in aged skin that allows longer time for the proteolysis of the filaggrin to be converted into NMF. [10,15] The aged epidermis of the forearm contains more free amino acids and more trans-Urocanic acid than the young epidermis. There is a decline with age of the SC urea content. [135] Urea is a component of sweat and contributes to the hydration of the SC. The SC urea content is significantly lower for women > 55 years of age for the upper inner arm site. [142] No age-related differences were detected within the volar forearm site. [142]

Age-related changes are not well defined for the SC lactate content, which is another sweat component. [135] Some studies report that it declines with age. The SC lactate content is significantly lower for women > 55 years of age in the upper inner arm site. [142] No age-related differences were detected within the volar forearm site. HA has recently been shown to be present naturally in the epidermis and is considered to be part of the NMF. [14] The decrease of HA in intrinsically aged skin may also play a critical role for other age-related effects, including lower keratinocyte proliferation rate

and thinner epidermis. [143] HA is binding to the extracellular space via the protein CD43. [14,144] Oh et al., show that the epidermal water content is correlated with both HA and total sulfated Glycose Amino Glycans (tsGAG). [143]

The epidermis possesses an extremely efficient antioxidant system that is superior to most other tissues. [145] This system displays a decrease in efficiency as a function of skin aging. [146] Ascorbic acid levels are lower in the epidermis of both photoaged and aged skin, while α-tocopherol (known as vitamin E) is lower in the epidermis but not in the dermis. Other antioxidant molecules and enzymes such as Cu, Zn, super oxide dismutase (SOD), catalase, and glutathione peroxidase levels also decrease with age. [145,147]

The human epidermis plays a role in the generation of the active form of vitamin D, $1.25(OH)_2D_3$. Besides its role in calcium homeostasis and bone maintenance, vitamin D has been implicated in immune responses, affecting macrophage function and modulating the release of inflammatory cytokines. [148,149] Therefore, when the synthesis of vitamin D decreases, the immune response is also impaired. [150] Elderly people have reduced serum levels of vitamin D in their diet, which in part may be due to insufficient sun exposure.

Reduced cytokine production [151] and/or downstream reactivity in skin have been associated with aging. [152] Interleukin (IL)-1α and IL-1β are the major keratinocyte cytokines. They are normally expressed in all epidermal cell layers and barrier disruption markedly enhances both IL-1α mRNA and protein levels. In contrast aged epidermis expresses subnormal mRNA and protein levels of certain membersof this cytokine family. [138,154]

Extrinsic (Photo) Aging

Visual Appearance [109,155–157]

The term “photoaging” was used for the first time in 1986. It describes the effects of chronic UV light exposure on the skin. [158] Typically, individuals who follow outdoors lifestyles and live in sunny places are expected to have some degree of photoaging. During human life different body skin sites are exposed at varying levels. UV light can cause lipid peroxidation in the SC and DNA damage in the viable epidermis.

Long term effects of sunlight on skin are profound and it is estimated that they account for up to 90% of visible skin aging [159], particularly in people without the natural protection associated with higher levels of melanin content in the skin. [160] Quantitative and qualitative photoaging can remain invisible for decades before clinical signs become evident. The changes induced by chronic sun exposure can occur well before visible signs of chronological aging and depend on skin type, nature of sun exposure (occupational vs recreational), hair style, and dress.

The effects on the skin following exposure to UV radiation include changes in surface texture [161], early appearance of dyschromia and lentigines, increased roughness, and the development of fine rhytides. [161]

Thickness of the SC and the Epidermis

Corneocytes of photoaged skin become polymorphic with increasing abnormalities: retention of nuclear remnants and roughening of border edges. [121,162] The epidermal thickness of exposed skin is modified. Extrinsic aging shows an irregular thickening of the epidermis: [5] the epidermal thickness increases (corresponding to hyperplasia) then decreases (corresponding to severe atrophy). It is unlikely that UV-

induced effects play a major role in a decrease of epidermal thickness with age. [73]

Some authors found an increase of the SC thickness of the exposed skin site. [71] With respect to UVB-induced erythema the SC thickness appears to be a more efficient shield for photoprotection than either the extent of pigmentation or the total thickness of viable epidermis. [163]

Barrier Function

The superposition of photoaging and aging further aggravates barrier abnormality. [118] Skin Surface Lipids (SSL, squalene in particular) which coat the surface of sebaceous gland-enriched skin sites, such as the face, are first-line targets of UV light. Irradiation of squalene with UVB and UVA leads to formation of squalene hydroperoxide [164–166] resulting in oxidative stress. Photoaging is associated with the development of benign and malignant sebaceous tumors often located on the head and neck area. [167] Sebaceous gland hyperplasia develops mainly in ageing facial skin. However, sebaceous glands hyperplasia also occurs in the oral mucosa of elderly person where sunlight is not a relevant issue. [109]

SC Water Content

Gniadecka et al., found that photoaged skin has increased levels of SC water content observed by Raman Spectroscopy. [129] This is paradoxal as the aged skin surface is often dry. Boireau et al found using *in vivo* confocal Raman microspectroscopy a decrease of the SC water content on photoaged skin. [71] However, the changes are small.

Biochemical Composition

UV irradiation (<320 nm) converts the epidermal precursor of vitamin D_3 and dehydrocholesterol to previtamin D_3, which subsequently isomerizes to form vitamin D_3. With aging the levels of the epidermal precursor of vitamin D_3 decrease contributing to a decrease in vitamin D_3 production in older populations. [168] This can result in vitamin D3 deficiency in the absence of regular sun exposure. [169] Vitamin A is destroyed by sun exposure.

Following chronic UV exposure the levels of oxidatively modified proteins significantly increase. [170,171] UV filtration by the SC and the greater antioxidant capacity of the epidermis protect to some extend the nucleated epidermal layers. Remarkably catalase protein and activity levels are naturally present at a very high concentration in human SC and become significantly depleted upon UV exposure *in vivo.* [170,172] Hellmans et al., [172] showed that catalase levels and activity are strongly affected by UVA but remain unchanged upon UVB exposure. Recovery of the catalase enzyme after UV exposure occurred in 3-4 weeks following an age-depend fashion. Recovery is faster in younger individuals than in older. [173]

UV radiation stimulates and activates various cells to produce and release cytokines that may play a significant role in the process of photoaging. [174]

		Intrinsic aging	**Extrinsic (photo)aging**
STRUCTURE	SC / Epidermal Thickness	SC thickness: Marks [69] and Lock-Andersen et al., [70] reported no change in SC thickness, Boireau et al., [71] showed an increase of the SC thickness. Viable Epidermal thickness: Lavker & Kligman [72] and Lavker [73] and Branchet et al., [74] showed a tendency to decrease of the epidermal thickness with a rate that accelerates with aging. The resistance to pressure is also diminished. [74,79] Epidermal thickness: El-Domyati et al., [67] and Whitton & Everall. [68] found a constant epidermal thickness with aging.	Exposed epidermal thickness: Gilchrest [5] reported an irregular thickening of the epidermis, increase and decrease of the thickness. For Lavker [73], UV-induced effects play a major role in the decrease of epidermal thickness with age. Boireau et al., [71] found an increase of the SC thickness with aging of the exposed skin site.
FUNCTION	Barrier Function	TEWL: Ghadially et al., [80] and Roskos & Guy [81] reported no change in TEWL rates with aging; the SC barrier function is not altered significantly. Boireau et al., [71], Tagami [10], Berardesca & Maibach [84], Cua et al., [85], Kobayashi & Tagami [86] found that the SC barrier function evolves with aging. Lipid content: Cua et al. [88] and Schreiner et al. [89] showed no relationship between age and lipid content, Boireau et al., [71] and Roskos & Maibach [90] reported a decrease of the SC lipid content with aging.	Aggravation of the barrier abnormality with superimposition of both effects (intrinsic and extrinsic aging) showed by Reed et al. [118]
		Cua et al., [88] reported that the cholesterol, fatty acid and ceramides contents are constant throughout life. Rogers et al., [91], Boireau et al., [71] and Makrantonaki & Zouboulis [97] showed the cholesterol content decreases on the face but its percentage is constant throughout life. Rogers et al., [91], Saint Léger et al., [92] and Roskos [96] showed that the percentage of fatty acid is constant with aging. Rogers et al., [91] showed that the ceramide content decreases by 30% with aging on the face, hands and legs. Denda et al., [93] found that according to the time of life, some subclasses of ceramides increase, and some subclasses of ceramides decrease.	

		Intrinsic aging	Extrinsic (photo)aging
		Lipid structure: Aging is accompanied to the loosening of the organizational order and a decrease in the ordered inter-molecular structure according to Tfayli et al. [107] There is also a decrease of the trans/gauche conformation structures in the alkyl chain of lipids in the older skin indicating a lower intra-chain order and inducing an increase in the lateral packing disorder. [107] According to Boireau et al., [71] there is a decrease of the lipid compactness on the exposed arm site and on the protected arm site with aging.	
		Skin Surface lipids: Increase of the SSL content with age except on the ankle according to Cua et al., [88] and Saint Léger et al. [92] Sebaceous glands: The size of sebaceous glands varies with aging. Le Fur et al., [108] showed that the level of sebaceous glands in the on the face of elderly is similar to those of pubertal children. In aged skin the turnover of the sebaceous glands is slowed down compared to the young skin. Sebum: The sebum production declines with age pH: According to Waller & Maibach [77] the pH is constant until 70 years old and then rises, except on facial skin where the pH is more alkaline in aged subjects. Calcium: According to Denda et al., [117] the altered calcium gradient with aging accounts for the barrier abnormality. Barrier recovery: Ghadially et al., [80] showed that it takes 3 days in young population to recover the barrier and 6 days in old population. Topical substances are consequently absorbed more slowly in old people than young people.	

		Intrinsic aging	**Extrinsic (photo)aging**
	SC water content	Eisner et al., [122], Wilhelm et al., [83] and Cua et al., [85] reported no change of the global SC water content with age while Jackson et al. [7], Mc Callion & Po [8], Harvell & Maibach [75], Walrafen [124] and Boireau et al., [71] reported a slightly decrease of the SC water content with age. According to Gniadecka et al., [129] there is no alteration of the water structure with aging.	Increase of the SC water content with age, and paradoxally aged skin surface is often dry and weathered according to Gniadecka et al. [129] No alterations of the water structure. [129] Boireau et al., [71] found a decrease of the SC water content on the exposed arm site with aging
	Mechanical	The corneocytes become bigger due to a decreased epidermal turnover leading to roughness of the skin. [121,136]..	The corneocytes become polymorphic with roughening of border edges. [121,162]
	Cellular proliferation	The epidermal cell proliferation decreases by about 50% from 20 to 70 years old but number of cells remain stable. [121] Tagami [10] found that the SC is replaced by 15-20 days in young skin, while Grove & Kligman [95] and Baker & Blair [140] found that the SC is replaced in 40-60 in old skin.	The response to chronic UV exposure depends to the individual's skin type. Changes in cell number are also prominent in sun-exposed skin beginning in childhood.
COMPOSITION	Biochemical composition	NMF: Mc Callion & Po [8] showed that there is a decline in NMF production. Elderly skin of the cheek and the forearm contain more NMF than in the young skin. Decline of urea content according to Egawa & Tagami. [135]	Vitamin: Decrease of vitamin D3 in older population, destruction of vitamin A by sun exposure. [168]
		Even if the decline of the SC lactate content with age is not so well-defined, there is a significantly lower lactate content for women 55 years old and over in the upper inner arm site according to Wu & Kilpatrick-Liverman [142] Decrease of hyaluronic acid content according to Oh et al., [143] Decrease of efficiency of antioxidant activity [146]	

Conclusion

In this review the main effects of chronological and extrinsic aging on the SC are highlighted, summarized in Table 1. The observed effects depend on the used method, the body skin sites and the groups of age. Intrinsic aging and extrinsic aging lead to visible and non-visible modifications of the epidermal skin at tissue, cellular and molecular level. Some changes are the results of cell senescence, while others are linked to environmental insults such as UV radiation, exposure to temperature extremes or stress. The age-related modifications that are observable macroscopically can be attributed to changes that happen at the molecular, supramolecular, and cellular level.

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ETUDES EXPÉRIMENTALES

CHAPITRE:

2 FONCTION BARRIÈRE ET VIEILLISSEMENT CUTANÉ

I FONCTION BARRIÈRE

Changements dépendants de l'âge et du site corporel sur la fonction barrière cutanée

1 | Contexte

Le vieillissement cutané et le photo-vieillissement sont souvent associés à des modifications protéiques au niveau du derme. La diminution du collagène et la modification de l'élastine entrainent l'apparition de rides (Takahashi et al. 1989), une perte d'élasticité (Gilchrest 1996; Brincat et al. 2005) et de tonicité, ainsi qu'un teint terne. Le vieillissement cutané et le photo-vieillissement (Uitto 1997) impactent également l'épiderme au niveau des couches supérieures de l'épiderme, *Stratum Corneum* ou couche cornée. La fonction barrière cutanée dépend principalement de la structure tissulaire du SC avec notamment l'épaisseur de la couche cornée (Tagami 2008; Machado et al. 2010), de la teneur en lipides intercellulaires (Downing and Stewart 2000) notamment des céramides et du cholestérol et enfin de l'organisation supramoléculaire de ces lipides (Pilgram et al. 1999) formant le ciment lipidique intercornéocytaire. L'eau contenue dans le SC induit des intéractions moléculaires de type liaison H en particulier au niveau des têtes polaires céramidiques agissant ainsi sur l'organisation supramoléculaire des lipides. Dans ce travail, nous avons étudié les effets *in vivo* du vieillissement chronologique et du photo-vieillissement sur la fonction barrière cutanée selon différents sites corporels par l'utilisation d'une méthode spectroscopique vibrationnelle en plein essor : la micro-spectroscopie confocale Raman. Les effets de ces deux types de vieillissement sur la barrière cutanée ont été reliés aux variations des paramètres physiologiques énoncés : épaisseur de la couche cornée, teneur en lipides et organisation lipidique de la couche cornée.

2 | Méthodes

Des mesures *in vivo* à Température et Humidité Relative contrôlées (20-25 °C, 40% RH) ont été effectuées sur trois sites corporels : joue, bras exposé au soleil et bras photo-protégé, sur un groupe de femmes caucasiennes réparties selon quatre groupes d'âge distincts de 18 à 70 ans. Les effets du vieillissement chronologique ont été étudiés sur le SC du bras face antérieure (protégé) et du photo-vieillissement sur le SC de l'avant-bras face postérieure (exposé). Enfin, les effets du vieillissement cutané et du photo-vieillissement ont été étudiés sur la joue, partie corporelle qui a un comportement physiologique cutané différent et qui est l'objet de soins cosmétiques particuliers et plus fréquents que ceux effectués sur le reste du corps. Les mesures ont principalement été réalisées en micro-spectroscopie confocale Raman sur les trois sites corporels et à différentes profondeurs dans la couche cornée (de la surface de la peau à la jonction couche cornée-couche granuleuse). Au niveau tissulaire, l'épaisseur de la couche cornée peut être obtenue de deux façons différentes : par étude de l'évolution de la bande de vibration CH_2 des lipides (autour de 2850 cm^{-1}), ou à partir des profils de concentration de l'eau depuis la surface jusqu'à la jonction SC-SG. L'épaisseur de la couche cornée a donc ici été déterminée à partir du profil de concentration de l'eau (Egawa et al. 2007; Egawa and Tagami 2008; Bielfeldt et al. 2009). Chaque profil de concentration de l'eau est obtenu graphiquement en représentant le ratio desintensités de la bande de vibration de la liaison O-H entre 3350-3550 cm^{-1} et la bande de vibration de la liaison CH_2 des protéines entre 2910-2965 cm^{-1}, en fonction de la profondeur de chacune des mesures spectrales. Il se présente comme une fonction logarithmique atteignant un plateau après une augmentation linéaire. L'épaisseur du SC est calculé par la méthode des tangentes. En effet, le SC est différent selon la personne et le site corporel.

Au niveau moléculaire, la teneur en chacun des composés étudiés (teneur relative en lipide, teneur totale en cholestérol et en céramides exprimés en [1]céramide 3) est calculée pour chaque profondeur (**Figure 4**):

- à partir du descripteur Lipides/Protéines, L/P (**Figure 5**): ratio de l'intensité des bandes de vibration de la liaison C-H des lipides sur l'intensité des bandes de vibrations de la liaison C-H des protéines, $\nu_{\text{bandes de vibration des CH2 lipides}}/\nu_{\text{bandes de vibration des CH2 protéines}}$, avec :

  $\nu_{\text{bandes de vibration des CH2 lipides}}$: (2820-2900) cm^{-1}

  $\nu_{\text{bandes de vibration des CH2 protéines}}$: (2910-2965) cm^{-1}.

- à partir d'algorithmes mathématiques développés par River Diagnostic pour le cholestérol (Caspers et al. 2001) et les céramides (Caspers et al. 2001, 2003)

[1]Mélange commercial de céramides et d'acides gras vendu par Sigma-Aldrich et qui n'est pas complètement défini

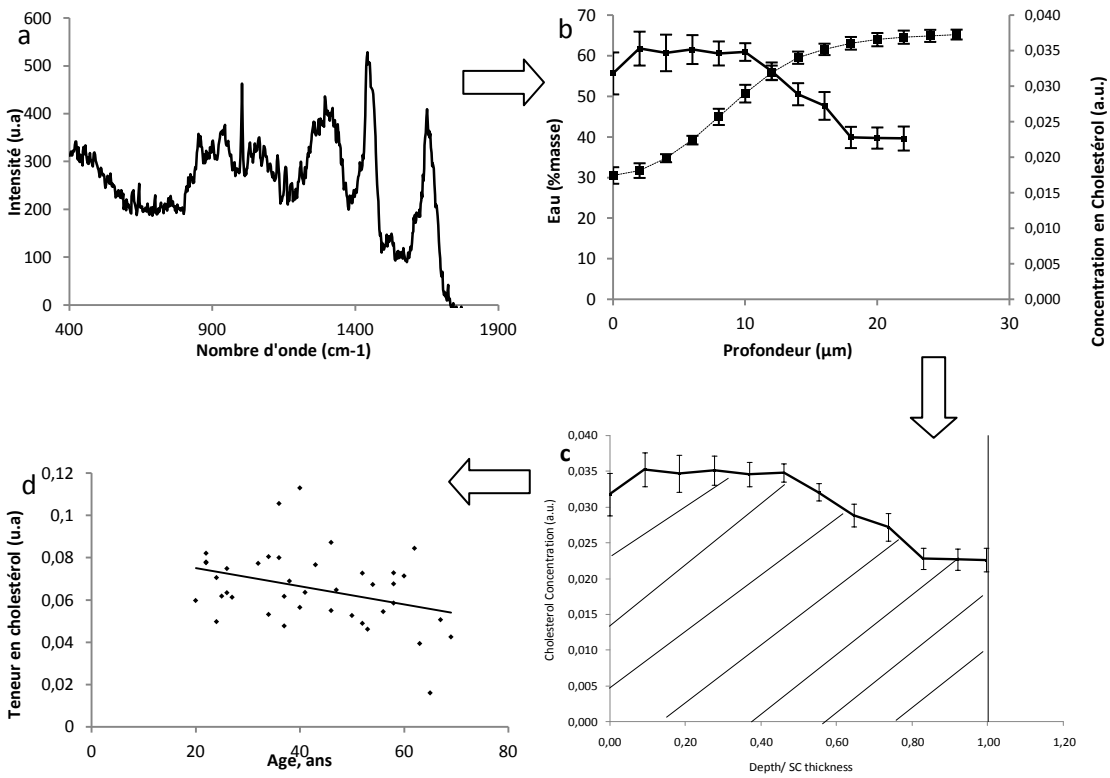

Figure 4 : Détermination de la teneur d'un composé *(ici en exemple le cholestérol)* ***à partir de son profil de concentration****. Tout d'abord le a) Spectre brut de la peau est obtenu par micro-spectroscopie confocale Raman. Le b) profil de concentration peut être alors représenté graphiquement, et le c) profil de concentration normalisé. Enfin, la somme sous la courbe du graphique de l'intensité du signal en fonction de la profondeur permet de construire le d) graphique teneur du composé en fonction de l'âge.*

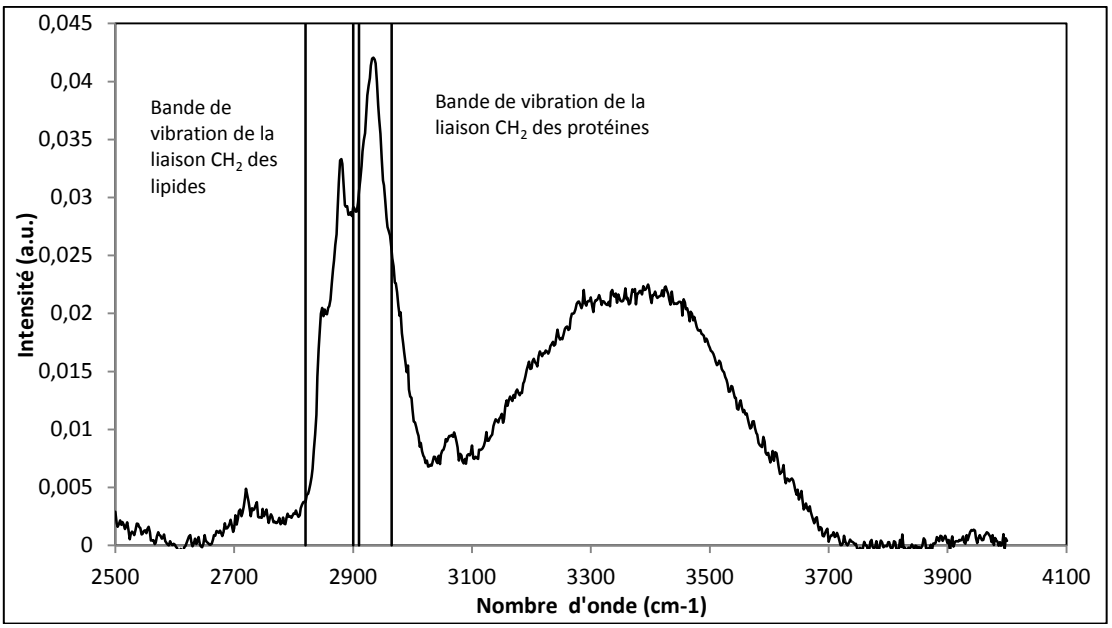

Figure 5: Bande spectrale de vibration de la liaison CH_2 des lipides et de la liaison CH_2 des protéines dans la région 2500 – 4000 cm^{-1} *avec la bande caractéristique de la liaison CH_2 (2820-2900 cm^{-1}) des lipides et la bande caractéristique de la liaison CH_2 des protéines (2910-2965 cm^{-1}).*

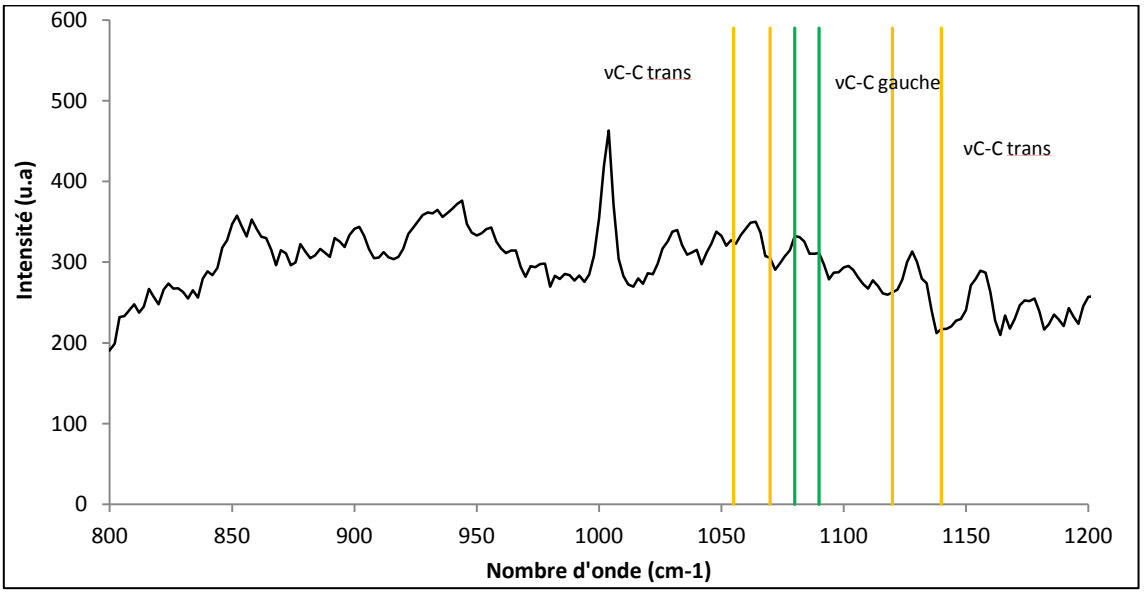

Figure 6: Bande spectrale de la liaison C-C gauche et C-C trans dans la région 400 - 1800 cm^{-1}, *avec $\upsilon_{C\text{-}C\ trans\ conformation\ 1}$:(1055-1070) cm^{-1}, $\upsilon_{C\text{-}C\ trans\ conformation\ 2}$:(1120-1140) cm^{-1}, et $\upsilon_{C\text{-}C\ gauche\ conformation}$:(1080-1090) cm^{-1}.*

La teneur totale en chacun des composés est ensuite obtenue par intégration des profils de concentration précédents depuis la surface du SC jusqu'à la jonction entre le SC et le SG. En parallèle, les profils de concentration des composés sont représentés graphiquement en normalisant la profondeur de 0 (surface) à 1 (jonction SC-SG) pour analyser les résultats intra- et inter-groupes.

L'organisation des édifices lipidiques a fait l'objet d'une investigation à partir d'un paramètre spectroscopique traduisant la compacité des édifices lipidiques du ciment intercornéocytaire (**Figure 6**) : le rapport d'intensité des bandes de vibration de la liaison C-C trans et de la liaison C-C gauche, $\nu_{\text{C-Ctrans conformation(1+2)}}/\nu_{\text{ C-Cgauche conformation}}$ avec :

$\upsilon_{\text{C-C trans conformation 1}}$:(1055-1070) cm^{-1}

$\upsilon_{\text{C-C trans conformation 2}}$:(1120-1140) cm^{-1}

$\upsilon_{\text{C-C gauche conformation}}$:(1080-1090) cm^{-1}

En addition, des mesures biométriques ont été réalisées, en particulier la mesure de la Perte Insensible en Eau, PIE, qui caractérise classiquement et de façon globale la qualité de la fonction barrière cutanée (Elias 2005).

3 | Résultats

Notre étude exhaustive intègre à la fois un critère global de caractérisation de la fonction barrière (PIE) et une explication aux niveaux tissulaire, moléculaire et supramoléculaire du vieillissement cutané chronologique et du photo-vieillissement. La diminution générale avec l'âge de la PIE sur les deux sites du bras, bras protégé et bras exposé, a caractérisé une légère amélioration de la fonction barrière cutanée en vieillissant. L'épaississement de la couche cornée observé sur ces sites corporels avec l'âge explique en partie cette diminution de PIE. Il résulte lui-même de la diminution de

la desquamation (Rawlings 2010) lié au vieillissement naturel. Au niveau moléculaire, la teneur relative en lipides totaux diminue significativement avec l'âge sur les deux sites du bras avec une évolution similaire. De même,la teneur en céramides diminue avec le vieillissement, ce qui est consistant avec une diminution de l'activité enzymatique synthétisant des céramides (Jensen et al. 2005). Ces teneurs diminuent légèrement avec l'âge mais elles sont compensées par l'augmentation de l'épaisseur de la couche cornée. Au niveau supramoléculaire, seul le photo-vieillissement impacte la compacité lipidique car seul le SC du bras exposé est moins compact en vieillissant, le rapport $\nu_{C\text{-}C\text{trans conformation}}/\nu_{C\text{-}C\text{gauche conformation}}$ diminuant avec l'âge.

De façon parallèle, à tout âge, le SC de la joue a une fonction barrière plus faible en comparaison avec la fonction barrière des deux autres sites corporels, site protégé et site exposé du bras: la PIE est plus grande sur la joue. Cette fonction barrière s'améliore légèrement avec l'âge. La joue est caractérisée par une épaisseur plus fine du SC que les deux autres sites corporels. Son épaisseur augmente plus lentement par rapport à l'épaisseur des deux autres sites corporels. En effet, le renouvellement cellulaire épidermique est plus rapide sur la joue que sur les sites du bras et conduit à une taille de cornéocyte plus petite (Plewig 1970; Stamatas et al. 2006). De plus, la desquamation est plus rapide sur la joue que sur les bras protégé et exposé. Cette épaisseur fine est compensée par des niveaux importants de cholestérol et de céramidesdans le SC de la joue quelque soit l'âge. Seule la teneur en cholestérol diminue légèrement avec l'âge sur la joue mais reste toutefois supérieure à la teneur dans la couche cornée des deux sites du bras. Il est intéressant de noter que malgré le comportement physiologique cutané différent de la joue par rapport au reste du corps, le SC de celle-ci se comporte de la même façon que les autres sites corporels.

4 | Conclusion

L'intégrité de la fonction barrière cutanée dépend à la fois de l'âge et du site corporel. Globalement, il n'y a pas de perturbations fondamentales de la qualité de la fonction barrière cutanée. Le vieillissement cutané et l'exposition à l'environnement impactentla structure et la composition de la couche cornée. La diminution ainsi que l'affaiblissement de la compacité du ciment lipidique sont compensés par l'augmentation de l'épaisseur de la couche cornée. Finalement, le vieillissement cutané a un impact sur l'épaisseur de la couche cornée alors que le photo-vieillissement a un impact sur la teneur en lipides et la compacité lipidique.

II ARTICLE 1

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Age-dependent changes in stratum corneum barrier function

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Abstract

Background/purpose The Stratum Corneum (SC) barrier function mainly depends on the SC structure at the tissue level, its composition, and the organization of intercellular lipidic cement at the molecular level. The goal of the present study was to assess the age-dependent changes of the SC barrier function and the associated physiological parameters.

Methods This study was conducted on 40 French women divided into 4 groups of age. Measurements were done on three sites: cheek, protected and exposed arm sites. SC composition (water, lipid/protein ratio, cholesterol, ceramides) was measured using Raman confocal microspectroscopy, skin surface hydration using skin conductance, and barrier function through trans-epidermal water loss (TEWL) measurements.

Results TEWL decreases slightly with age, which is partially explained by the age-dependent increase of the SC thickness. This decrease is faster for the face compared to both arm sites. The lipid to protein ratio and lipid compactness decrease significantly with age only for the arm sites. Water concentration profiles only decrease very close to the skin surface. At all ages tested, the SC on the cheek showed significantly higher TEWL, water and lipid content and less thickness compared to the arm sites. Comparison of the exposed to unexposed arm site showed difference only for the lipid compactness at the older group studied.

Conclusion Skin aging, body site and environmental exposure can affect the SC barrier function, its structure, and its lipid content. The thickening of the SC with age compensates for the decrease of the quantity and ordering of the lipidic cement.

Introduction

Skin appearance is changing with age: for example, emergence of wrinkles (1), loss of elasticity (2,3) and reduction of moisturization. The skin ageing process has been attributed to intrinsic and extrinsic factors, referred to respectively as chronological aging and photoaging (4). Initially, skin ageing has been studied in relation to benign, premalignant and malignant neoplasms (5). An increasing interest in preserving a youthful skin appearance reflecting good health (6) shifted the focus to other manifestations of skin ageing such as coarseness, wrinkling, sallow discoloration, telangiectasia, and irregular pigmentation and their underlying factors.

One of the most important functions of the skin is to protect the body against dehydration and against environmental insults such as sun exposure, variations of humidity and temperature, wind and chemical irritants. The quality of this barrier function is typically assessed by measuring the transepidermal water loss (TEWL). Higher TEWL indicates less efficient barrier function (7).

The skin barrier function is mainly conferred by the top layer of the epidermis, the Stratum Corneum (SC). Three physiological parameters contribute to the quality of the barrier function: the SC thickness (8,9), the intercellular lipid content (10), and the lipid organization (11). The SC thickness depends on the balance between desquamation and replenishing of the corneocytes. The SC intercellular lipids are derived from the layer below the SC, the Stratum Granulosum (SG), as part of the keratinocyte differentiation process. The composition of the SC lipids includes free fatty acids, cholesterol, and ceramides. Lipid chains can take conformations (trans or gauche), which could affect the compactness of the intercellular lipid layers, which may influence water transport in the SC. Moreover, the lateral ordering of SC lipids is known to be important. There is a balance between orthorhombic and hexagonal packing (12). Both organizations are lamellar but an orthorhombic organization is necessary to let water to circulate through the lipid phase (13–15), while the percentage of the orthorhombic phase reduces the rate of water flux through the tissue. The size of the

corneocytes -linked by corneodesmosomes which play an important role in the cohesion of SC- (16) can affect the barrier function by creating a longer tortuous diffusion path.

In this work we investigated the age-dependent changes in SC barrier function with regards to the above mentioned parameters by using non invasive methods. SC thickness and lipid composition may differ from site to site and can be affected by chronic exposure to environmental elements. For this reason in this study we chose to investigate the age-related changes in skin barrier function on three sites: the face (cheek), an exposed arm site (dorsal forearm), and a protected arm site (upper inner arm).

Materials and Methods

a) Population

The study was conducted in accordance with the ethical principles of The Declaration of Helsinki. Healthy female volunteers of Fitzpatrick skin types I-III with no dermatological conditions participated in the study following signed informed consent. They were divided into 4 groups of 10 based on their age: G1: 18-30, G2: 30-40, G3: 40-55, and G4: 55-70 years of age. All measurements were performed following 15 min of acclimatization in an environmentally controlled room (20-25°C, 40% relative humidity). They did not expose themselves intentionally for a long time to the sun (or tanning beds) for at least one month before measurement and they have not used self-tanning products, which might interfere with the Raman measurements by introducing high background fluorescence. Data collection started in September and continued for two months. They were not allowed to use any skin care product or deodorant or have a warm drink the morning of the test. Non-invasive measurements were performed on three skin sites: face (central cheek area), relatively exposed arm site (dorsal forearm) and relatively protected arm site (upper inner arm).

b) In vivo measurements

The quality of the skin's barrier function was evaluated through TEWL using a closed chamber instrument (Vapometer, Delfin, Kuopio, Finland) (17). The average of 6 measurements was recorded for each volunteer and each skin site.

Skin surface hydration was evaluated through high frequency skin conductance using Skicon-200EX (I.B.S. Company, Ltd., Japan) (18). This method measures electrical properties relating to the hydration level of the upper SC. The average of 6 measurements was recorded for each volunteer and each skin site.

SC thickness, total SC water content, lipids content, lipid compactness, ceramide, and cholesterol levels were evaluated using confocal Raman microspectroscopy (Skin Analyser model 3510, River Diagnostics, Rotterdam, The Netherlands). A 671-nm laser was used to collect data at the high wavenumber spectral range (2600-3800 cm^{-1}) for the determination of water content and lipid to protein ratio. A 785-nm laser was used to collect Raman spectra in the fingerprint region (400-1800 cm^{-1}) from which cholesterol and ceramide levels were calculated as well as SC lipid compactness. In the high wavenumber region spectra were taken at depths through the SC from 0 to 32 µm every 4µm and in the fingerprint region from 0 to 24 µm depth every 4 µm. A total of 10 measurements were acquired in each wavenumber region at each site and for each volunteer. The Raman instrument was calibrated once at the beginning of each experiment day according to manufacturers' instructions. Before each measurement at a different skin site the CaF_2 window was cleaned up with a single wipe of tissue with a drop of methanol.

c) Spectral analysis

To evaluate the water content inside the SC expressed in g water per 100 g wet tissue (mass %), we used the intensity ratio of water /protein as described by Caspers et al. (19–22).

The SC thickness was calculated using the water profile concentration as described by

Bielfeldt et al.(23) and Egawa et al. (24,25). The average SC thickness of the 10 measurements of the water profile for each skin site and each volunteer was calculated. Then, from these values the average SC thickness for each site and each age group was used to normalize the depth values (x-axis) for the calculated concentration profiles. The total amount of a component in the SC was calculated as the integrated values between the normalized depths 0 (surface) and 1 (junction between SC and stratum granulosum).

The lipid to protein ratio was calculated as the ratio of the areas under the peaks corresponding to lipids (2820-2900 cm^{-1}) and protein (2910-2965 cm^{-1}) for each volunteer on each site.

Lipid compactness was calculated from the Raman spectra in the fingerprint region (400-1800 cm^{-1}) by measuring the area under three Raman bands corresponding to first C-C trans conformation (1055-1070 cm^{-1}), second C-C trans conformation (1120-1140 cm^{-1}), and C-C gauche conformation (1080-1090 cm^{-1}). The relative proportion of the trans conformation of intercellular lipids is an indicator of the lipid lateral compactness. The ratio trans to gauche is given by the intensities of $I_{1055\text{-}1070}+I_{1120\text{-}1140}/I_{1080\text{-}1090}$ (26).

The spectrum of ceramide type 3 was used as one of the primary spectra for fitting the Raman data in the SC (22) and was considered as a surrogate marker for total ceramide content. The concentration of cholesterol relative to the keratin signal was also calculated from the Raman spectra in the region 400–2200 cm^{-1} using a previously reported algorithm (21).

d) Statistical analysis

Linear regression versus subject age was tested for each of the following parameters: total SC water, lipid to protein ratio, cholesterol content, ceramide content, SC thickness, and lipid compactness. For each distribution the regression coefficient R^2 and the significance of the correlation p were calculated. For each distribution mean +/- one standard error of mean are shown in the graphs. Statistical comparison of two

distributions was performed following the Anderson-Darling normality test and test of variance (F-test) in order to select the appropriate t-test. Statistical significance was accepted at the level of α=0.05.

Results

The function, structure, and molecular composition of SC were found to depend on the subject age and the skin site investigated. In the following paragraphs we examine these relations.

a) The integrity of the SC barrier function depends on age and on skin site

TEWL measured on the face was found to be higher than on the two arm sites confirming previous reports (27,28). There was no statistical difference for the values between the two arm sites. Linear regression of TEWL vs. age showed a general decrease with advancing age on all skin sites tested (Figure 1). The decrease of the TEWL values is significant for the face and for the protected arm site ($p< 0.05$), indicating a gradual strengthening of the barrier function with age on these two body skin sites. Moreover the rate of change of TEWL with age on the face is approximately twice higher than that on the protected arm site.

Figure 1

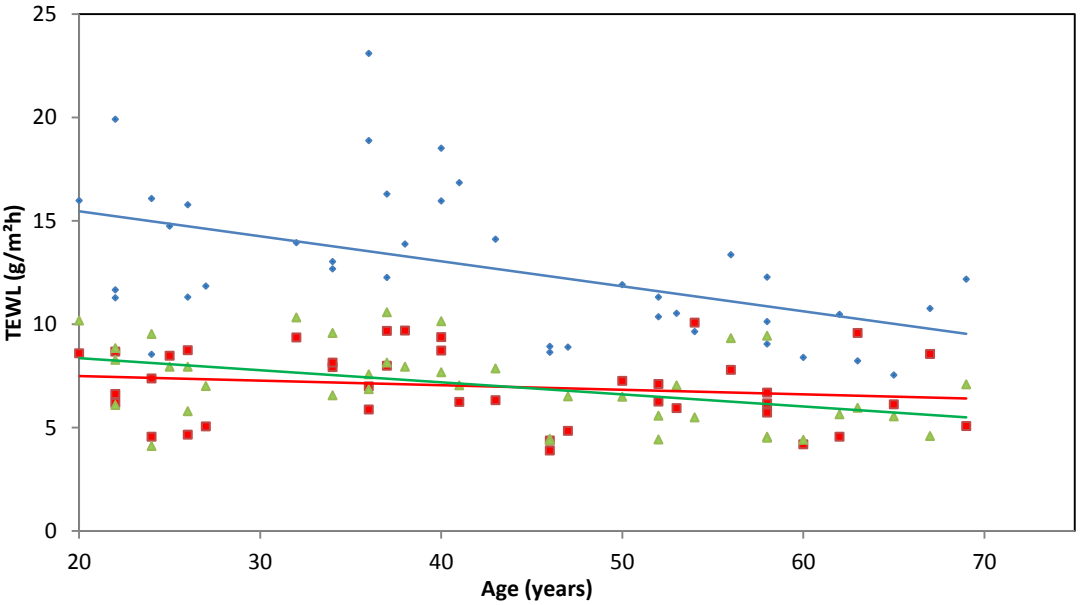

Fig 1. Skin barrier function changes with age and skin sites. *Individual data points are shown for face (cheek, blue diamonds), dorsal forearm (red squares), and upper inner arm (green triangles). Significant correlation was found for the face (y=-0.120*x+17.887, R^2=0.244, p=0.001) and for the upper inner arm (y=-0.0584*x+9.53, R^2= 0.197, p <0.05). For the dorsal forearm no significant correlation was found.*

b) SC Thickness depends on age and skin site

The SC thickness on the face was found to be thinner than on the two arm sites. This observation is in accordance with Tagami (8) who described about 10 cell layers in the SC of face and about 15 cell layers for the body. The SC thickness is not statistically different between the two arm sites for all groups of age. Linear regression of SC thickness vs. age showed a significant increase with aging on all skin sites tested, p< 0.05 (Figure 2) indicating a thickening of the SC. Moreover the rate of the thickness increase with age for both arm sites is almost the same. This means that the increase of the SC thickness is more related to chronological aging rather than an extrinsic effect (e.g. photo aging). Interestingly, individual measurements are more dispersed for the

population with ages more than 50 years compared to the younger groups. This observation could be attributed to the individual history for each volunteer: cumulative sun exposure, smoking, stress, and cosmetic habits.

Figure 2

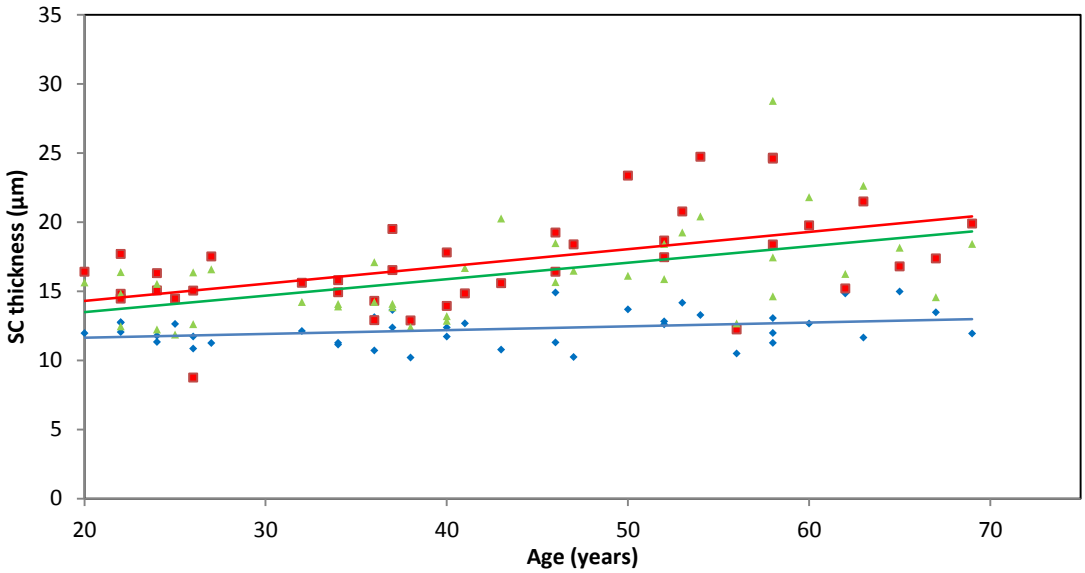

Fig 2: SC thickness depends on age and skin site. *Individual data points are shown for face (cheek, blue diamonds), dorsal forearm (red squares), and upper inner arm (green triangles). Significant correlation was found all body skin sites: face (y=0.0273*x+11.1, R^2=0.104, p=0.043), the upper inner arm (y=0.101*x+11.6, R^2= 0.294, p <0.001) and the dorsal forearm (y=0.125*x+11.8, R^2=0.276, p=0.001).*

c) The SC intercellular lipid content and the lateral compactness of SC lipids depend on age and skin site

The lipid to protein ratio in the SC on the face was found to be higher than on the two arm sites. There was statistical difference for the lipid to protein ratio values between the two arms sites only for the oldest group tested. Linear regression of lipid to protein ratio versus age showed a general decrease with aging on all body skin sites tested (Figure 3). The decrease of lipid to protein ratio values with age is significant for the two arm sites, $p< 0.05$. Moreover as in the case of SC thickness, the rate of change of lipid to protein ratio with age on the two arm sites is almost the same.

Figure 3

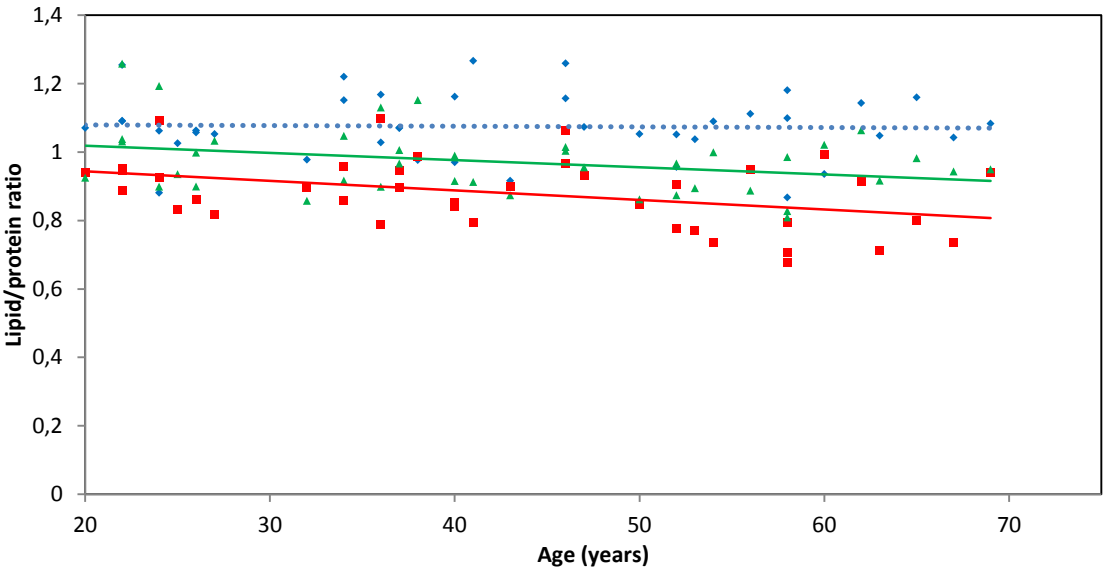

***Fig 3: Lipid to protein ratio depends on age and skin site**. Individual data points are shown for face (cheek, blue diamonds), dorsal forearm (red squares), and upper inner arm (green triangles). Significant correlation was found for the two arm sites: the upper inner arm (y=-0.00209*x+1.06, R^2=0.100, p=0.046) and the dorsal forearm (y=-0.00278*x+0.999, R^2= 0.155, p =0.013). No statistical difference was found for the face.*

Linear regression of the SC lipid compactness vs. age showed no variation with aging except for the exposed arm site (Figure 4). There was statistical difference for the SC lipid compactness, only for the oldest group, between the values for the exposed arm site and those for the face or the protected arm site.

Figure 4

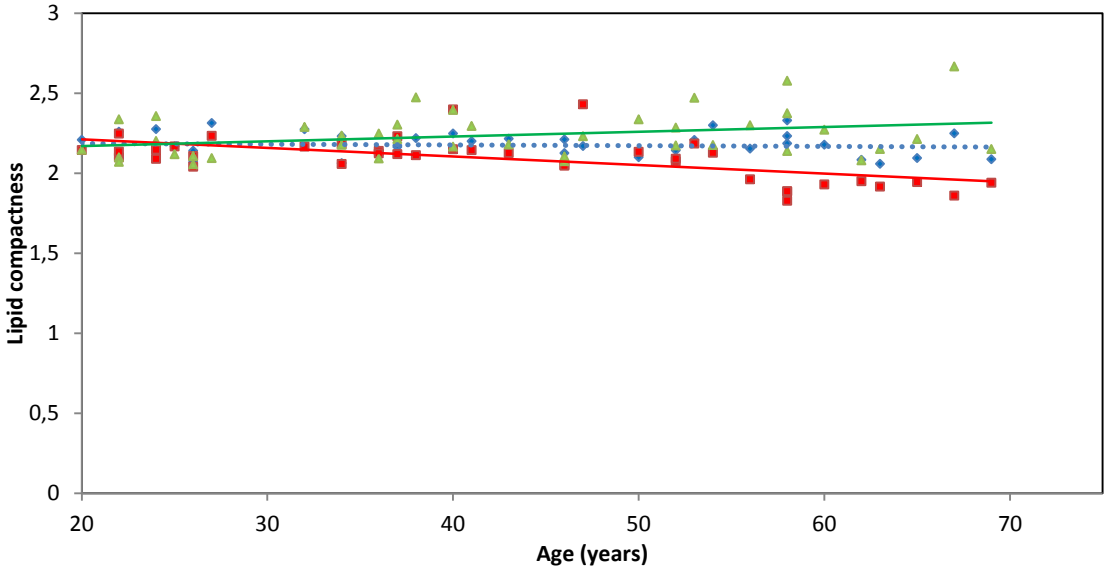

Fig 4: Lipid compactness ratio does not depend on age and skin sites except for the oldest people on exposed arm site. *Individual data points are shown for face (cheek, blue diamonds), dorsal forearm (red squares), and upper inner arm (green triangles). Significant correlation was found the dorsal forearm (y=-0.00538*x+2.32, R^2= 0.355, p <0.001). No statistical difference was found for the face or the upper inner arm.*

d) SC molecular composition evolves with age and skin sites

The values for the SC total water content, cholesterol content, and ceramide content at the sites tested and their correlation statistics versus age are shown on Table 1. All 3 parameters were found to have higher values on the face than on the two arm sites. For the SC total water content and ceramide content, there was no statistical difference for the respective values between the two arm sites (except for the youngest group where the value of the ceramide content was statistical different between the exposed arm site and the protected arm site). Concerning the value of the SC cholesterol content there was a statistical difference between the two arm sites; the protected arm site showed higher cholesterol content than the exposed arm site regardless the age. Linear regression of these 3 parameters versus age showed a general decrease on all the three skin body sites. Only on the face the SC ceramide content does not change significantly with age. On the exposed arm site the 3 parameters decrease significantly

with age, $p<0.05$. On the face the SC cholesterol content decreases significantly with age, $p< 0.05$. And finally on the protected arm site the SC ceramide content decreases significantly with age. Interestingly the rate of change for the SC ceramide content is almost the same for the two arm sites.

Discussion

The goal of this study was to assess the age-dependent changes of the SC barrier function and the associated physiological parameters. To that end an *in vivo* investigation was conducted on 40 European Caucasian women divided into 4 age groups from 20 to 70 years. Three skin sites of interest were compared: face (cheek), exposed arm site (dorsal forearm), and protected arm site (upper inner arm). The two arm sites were selected to explore potential differences between intrinsic and extrinsic skin aging effects. Facial skin is also exposed to the environment but it is physiologically and structurally different from the arm skin. Moreover, facial skin is of great psychological importance. Our data show that SC function, SC structure and SC composition depends on the age of the person and the location of the skin site.

Does the SC barrier function evolve with age?

We report that the TEWL rates are overall decreasing with age on the three skin sites of interest, which indicates that the SC barrier function is generally improving. This is in agreement with previously published reports (29–32). The TEWL decrease with age is significant particularly for the face and the protected arm site. The relevant increase of the thickness of the SC on the three skin sites of interest could explain in part this improvement of the SC barrier function. On the other hand the amount of lipids in the SC (as measured by the lipid to protein ratio) and the molecular organization of lipids (gauche/trans orientation) seem not to be involved in this age-dependent barrier

improvement. The ceramide content tends to decrease on two arm sites with age which is consistent with the decreasing of the ceramide-synthesizing enzymes activities (33). On the contrary we have showed that the ceramide level does not change significantly with age on the face, in agreement with the study of Egawa and Tagami (25) on Asian subjects and that of Rogers et al. on Caucasian subjects (34).

Does the SC barrier function depend on the skin site?

The SC barrier function was found to be weaker on the face (higher TEWL rate) compared to the two arm sites. Moreover the SC is thinner on the face than on the two other skin sites tested. On the face the epidermal cell proliferation rate is faster than on any other part of body skin site leading to smaller corneocytes cell size (35,36). This epidermal cell proliferation rate slows down with age resulting in an increased corneocyte size. Facial skin is also characterized by faster desquamation rates (leading to a decreased SC thickness) and higher sebum production compared to the other body sites. Skincare habits on the face are different than the rest of the body and this also may in part explain the observed differences in SC barrier function. The thinner SC on the face is compensated by higher levels of cholesterol, ceramides, and total lipids (as measured by the lipid to protein ratio) compared to the two arm sites independently of age. Egawa and Tagamialso showed the lipid to protein ratio was higher on the face compared to other body skin sites (25). Moreover facial skin is more hydrated than the two arm sites regardless the age, a likely result of the higher epidermal cell turnover rate. SC lipid compactness was found to be similar between the three sites tested.

Does chronic environmental exposure affect the SC barrier function?

When comparing the two arm sites, there was overall no significant difference with regards to the SC barrier function, the SC thickness, and the SC lipid composition (except for cholesterol levels that are higher on the protected arm site). Interestingly, even the rates of change of these parameters were very close for the two arm sites. This observation indicates that the age-dependent changes of these parameters are driven more by intrinsic than extrinsic factors. The only significant difference between the two arm sites was found for the SC lipid content and SC molecular organization only for the oldest group. This observation may indicate that accumulation of effects due to environmental exposure would result in less lipid amount and less compact organization, although these effects apparently did not influence the TEWL rates which were equivalent for the two arm sites, even for the oldest group.

***Table 1. Summary table of the parameters measured** and their dependence on age and skin site.*

Method	Parameters	Age	Site (face vs arm)	Exposure(exposed vs protected arm site)
TEWL	**Barrier function**	Decreases except exposed arm site	Higher on face	No difference
RAMAN	**SC Thickness**	Increases	Less on face	No difference
	Lipid Compactness	Decreases on exposed arm site and protected arm site	No difference	No difference except for the oldest group where protected arm site > exposed arm site
	[Lipid]/[Protein]	Decreases except on face	Higher on face	Protected arm site>exposed arm site for the youngest and the oldest groups
	[Cholesterol]	Decreases on face and exposed arm site	Higher on face	Protected arm site > exposed arm site
	[Ceramide]	Decreases on protected arm site and exposed arm site	Higher on face	No difference; except for the youngest group: protected arm site < exposed arm site
	[Total water]	No difference; only significant decrease on the exposed arm site	Higher on face	No difference
SKIN CONDUCTANCE	**Moisturization**	No difference	Higher on face	Exposed arm site > protected arm site for all groups except the youngest group

In conclusion, the integrity of the SC barrier function depends on both age and skin sites. Moreover SC composition and SC structure depend on the skin site but also the exposure to the environmental. Interestingly the decrease of the lipid to protein ratio and SC lipid compactness to be compensated by a concomitant increase in the SC thickness. These data show that while SC thickness is affected by intrinsic aging, SC composition and lipid compactness are affected by extrinsic aging processes.

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III VIEILLISSEMENT CHRONOLOGIQUE ET MICRO-HÉTÉROGÉNÉITÉ DES CÉRAMIDES

Impact du vieillissement chronologique sur la micro-hétérogénéité des céramides

1 | Contexte

Le SC est composé de cornéocytes imbriqués dans une matrice lipidique. Les lipides intercellulaires sont principalement composés de céramides, d'acide gras libres, et de cholestérol (Elias and Friend 1975; Elias 1981; Downing et al. 1987; Grubauer et al. 1989; Coderch et al. 2003; Madison 2003) dans des proportions équimolaires (Wertz et al. 1985; Norlén et al. 1998; Weerheim and Ponec 2001; Masukawa et al. 2008; Harding et al. 2010). Le maintien d'une fonction barrière cutanée efficace résulte en partie de l'équilibre entre ces trois familles de lipides (Janssens et al. 2011). Les lipides de la matrice intercornéocytaire contribuent à la fonction barrière cutanée et ils sont également impliqués dans les propriétés de rétention d'eau de par leur architecture multi-lamellaire complexe (Elias and Friend 1975; Imokawa and Hattori 1985; Imokawa et al. 1986). La composition de la matrice lipidique influence leur organisation lamellaire (Bouwstra et al. 2002; Babita et al. 2006; Mendelsohn et al. 2006; Boncheva et al. 2008; Smeden et al. 2011; Rabionet et al. 2014) et latérale (Bouwstra et al. 2002; Babita et al. 2006; Mendelsohn et al. 2006; Boncheva et al. 2008). En l'état actuel, la classification des céramides est décrite sous forme de 30 sous-classes de céramides potentiellement présentes dans l'espace extracellulaire du SC (Masukawa et al. 2008; Smeden et al. 2011; Rabionet et al. 2014). Les céramides sont composés d'une partie acide gras liée par une liaison amide à une base sphingoide. Dans le SC, cinq bases sphingoides différentes ont été récemment recensées définissant les classes céramidiques (Robson et al. 1994):

- S: Sphingosine dénommée S-CER
- dS: dihydroxysphingosine ou sphinganine dénommée dS-CER
- P: Phytosphingosine dénommée P-CER
- H: 6-hydroxy-sphingosine dénommée H-CER
- T: dihydroxysphinganine dénommée T-CER (t' Kindt et al. 2012)

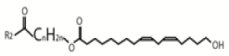
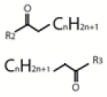
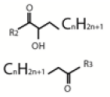
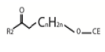
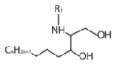
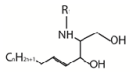
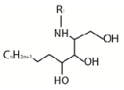
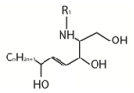
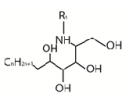
Cinq types d'acides gras et leurs liaisons aux bases sphingoides déterminant les sous-classes :

- N: chaîne d'acide gras non-hydroxylé/ non-estérifié
- A: chaîne d'acide gras α-hydroxylé, contenant un groupe hydroxyle
- EO: chaîne d'acide gras ω-estérifié, contenant notamment l'acide linoléique
- O: chaîne d'acide gras ω-hydroxylé liée à l'enveloppe cornifiée des cornéocytes (Breiden and Sandhoff 2014)
- 1-O-E: contient une chaîne d'acide gras supplémentaire, estérifiée avec un groupe hydroxyle OH primaire dans la base sphingoide

La nomenclature est basée sur la structure moléculaire des céramides. Motta et al. (Motta et al. 1993) et modifié ensuite par Robson et al. (Robson et al. 1994) ont désigné les classes et sous-classes de céramides par un minimum de 2 lettres. La première lettre indique le type d'acide gras attaché par la liaison amide (N, A, EO, O ou (1-O-E)). La dernière lettre définit la base sphingoide (S, dS, P, H ou T), voir le (**Tableau 1**).

L'objectif de cette étude est d'évaluer les effets potentiels de l'âge et le site corporel sur la composition lipidique du SC et particulièrement la composition des céramides.

Tableau 1: Tableau des céramides. *La nomenclature et la structure des céramides sont données dans le tableau. Chaque céramide est constitué d'une tête polaire (dS, S, P, H et T) ainsi que d'une partie acide gras (N, A, EO, O or (1-O-E)).*

	 N	 A	 EO	 (1-O-E)N	 (1-O-E)A	 O
 dS	CER(NdS)	CER(AdS)	CER(EOdS)	CER((1-O-E)NdS)	CER((1-O-E)AdS)	CER(OdS)
 S	CER(NS)	CER(AS)	CER(EOS)	CER((1-O-E)NS)	CER((1-O-E)AS)	CER(OS)
 P	CER(NP)	CER(AP)	CER(EOP)	CER((1-O-E)NP)	CER((1-O-E)AP)	CER(OP)
 H	CER(NH)	CER(AH)	CER(EOH)	CER((1-O-E)NH)	CER((1-O-E)AH)	CER(OH)
 T	CER(NT)	CER(AT)	CER(EOT)	CER((1-O-E)NT)	CER((1-O-E)AT)	CER(OT)

2 | Méthodes

Des mesures *in vivo* ont été réalisées sur deux groupes de femmes caucasiennes de 18-30 et 65-75 ans à Température et Humidité Relative contrôlées (20-25 °C, 40% RH) sur trois sites corporels: face, bras exposé au soleil et bras protégé du soleil. Des mesures biométriques ont été prises pour caractériser de façon globale la fonction barrière cutanée, la PIE. Des mesures en spectroscopie vibrationnelle (ATR-FTIR spectroscopie) ont été réalisées pour étudier au niveaux moléculaire et supramoléculaire les effets du vieillissement sur les lipides du ciment intercornéocytaire et plus précisément sur les céramides de la matrice lipidique. Les mesures permettent d'obtenir des informations dans les couches superficielles de la couche cornée (**Figure 7**) :

- la teneur en lipides par le ratio de l'intensité de la bande de vibration CH des lipides entre 2879 cm^{-1} – 2946 cm^{-1} et la bande de vibration CH des protéines ente 2946 cm^{-1} – 2975 cm^{-1}.
- l'organisation supramoléculaire des lipides en surface cutanée en étudiant le déplacement de la bande de vibration symétrique v_s CH_2 à 2848 cm^{-1}.
- la teneur en triglycérides obtenue par l'intensité de la bande carbonyle C=0 des esters lipidiques à 1740 cm^{-1} en lien avec l'importance du film hydrolipidique résultant des excrétions sébacées et des lipides obtenus après desquamation

Les informations spectroscopiques sont résumées dans le (**Tableau 2**).

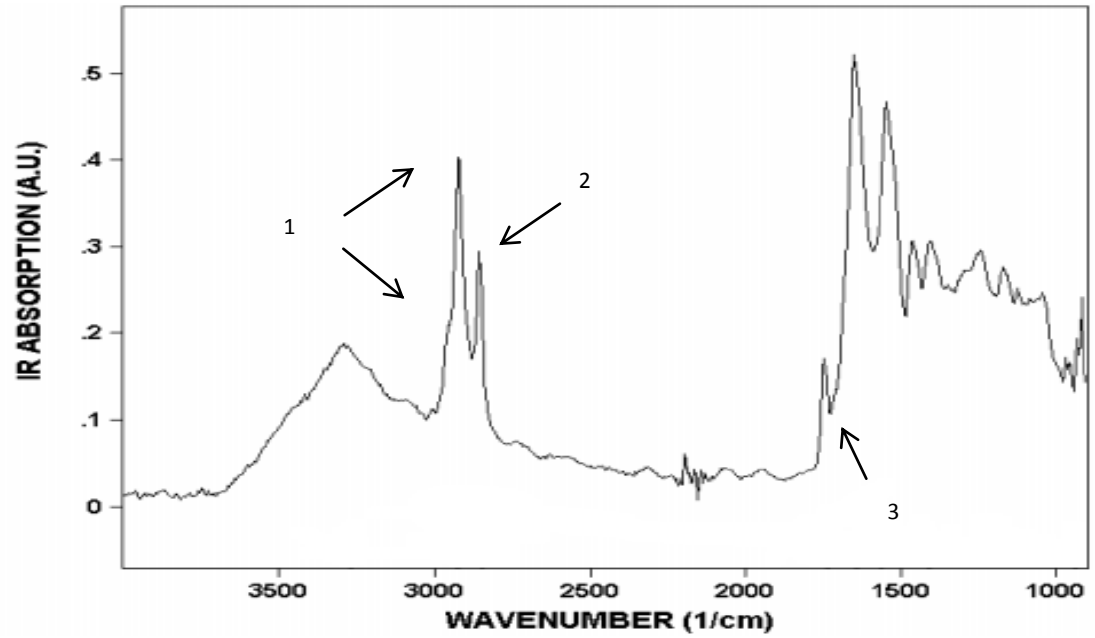


***Figure 7 : Descripteurs ATR-FTIR utilisés** pour décrire 1) le rapport lipides/protéines en utilisant $I_{2879\ -2946\ cm-1}/I_{2946-2975cm-1}$, 2) l'organisation lipidique par le déplacement de la bande de vibration symétrique $v_{s\ CH2}$ à 2848 cm^{-1}, et 3) la teneur en triglycérides en utilisant l'intensité de la bande carbonyle C=O des esters lipidiques à 1740 cm^{-1}. Adaptée d'après la publication de Nikiforos Kollias and Georgios N. Stamatas. Optical Non-Invasive Approaches to Diagnosis of Skin Diseases. JID Symposium Proceedings 7:64 -75, 2002.*

***Tableau 2: Informations pour l'analyse spectrale.** Tableau regroupant toutes les informations spectrales pour l'étude des lipides du ciment intercornéocytaire et des céramides en spectroscopie infrarouge.*

Méthodes	**Information**	**Analyse spectrale**	**Bandes**	**Ref**
ATR-FTIR	Lipide/protéine	Intensité de la bande de vibration CH des lipides	2879-2946 cm^{-1}	Kollias&Stamatas (Kollias and Stamatas 2002)
		Intensité de la bande de vibration CH des protéines	2946-2975 cm^{-1}	Kollias&Stamatas (Kollias and Stamatas 2002)
	Organisation Lipidique	Déplacement de la bande de vibration symétrique v_s CH_2	2848 cm^{-1}	Moore (Moore et al. 1997)
	Triglycérides	Intensité de la bande carbonyle C=0 des esters lipidiques	1740 cm^{-1}	Brancaleon (Brancaleon et al. 2000)

Des cotons-tiges après avoir été lavés de leurs impuretés ont été utilisés pour extraire les lipides cutanés sur chaque volontaire sur le site du bras protégé selon un protocole opératoire normalisé. Cette technique de prélèvement non invasive permet de diminuer les interférences dûes aux polymères observées avec la méthode des « tape-stripping ». Les lipides ont été extraits des cotons tiges par un mélange (Chloroforme : Méthanol, 2:1) puis repris après évaporation dans un mélange solvant (Chloroforme : Heptane, 1:9) contenant un étalon interne. Ce dernier permet de comparer les différents échantillons entre eux par comparaison des aires relatives $A_{céramide\ x}/A_{étalon\ interne}$. La comparaison des différentes classes et sous-classes de céramides est basée sur la normalisation interne. Cette méthode permet de semi-quantifier l'abondance des céramides de façon individuelle. Les échantillons contenant les lipides sont analysés par chromatographie liquide en phase normale couplée à un spectromètre de masse en haute résolution équipé d'une source d'ionisation chimique à pression atmosphérique, APCI, et d'un détecteur Orbitrap opérant en mode pleine échelle. La fragmentation est faite dans le mode HCD afin de mieux appréhender la structure des céramides. La HR-MS-APCI est utilisée en mode négatif et conduit à la formation d'adduits chlorés $[M+Cl]^-$. Les massifs isotopiques sont caractérisés par un pic plus abondant qui est sélectionné pour l'identification du composé. Ensuite, chaque chromatogramme est transposé en une matrice qualitative et quantitative. La matrice quantitative reprend pour chaque composé : l'adduit chloré $[M+Cl]^-$, m/z, temps de rétention, la formule brute, la classe, la sous-classe, le nombre total de carbone, le nombre d'insaturations de la chaîne alkyle, et l'intensité relative. Chaque céramide est défini par sa sous-classe, sa classe, le nombre de carbone et le nombre d'insaturations (exemple CER(NS) 50:0).

3 | Résultats

Les résultats par spectroscopie infrarouge ont montré que pour tout âge :

- $I_{lipides,\ joue} > I_{lipides,\ bras}$: les couches les plus superficielles du SC de la joue contiennent plus de lipides que les couches les plus superficielles du SC des deux autres sites corporels. Cette observation est également valable en profondeur, résultat observé par micro-spectroscopie confocale Raman observé précédemment (Boireau-Adamezyk et al. 2014).

- $I_{C=0,\ joue} > I_{C=O,\ bras}$: le film hydrolipidique est plus épais en surface sur la joue par rapport aux bras

Au niveau supramoléculaire, la spectroscopie infrarouge a montré que l'organisation latérale des lipides superficiels du SC était moins compacte pour la joue que pour les bras. Le signal IR à la surface provient de deux contributions, à savoir le signal provenant des édifices lipidiques superficiels du SC et le signal dû au film hydrolipidique. Ainsi, le signal du sébum qui a une organisation hexagonale (Michael-Jubeli et al. 2011) s'ajoute au signal des lipides des couches les plus superficielles du SC et conduit à un déplacement de la bande $\nu_s\ CH_2$ vers 2849 cm^{-1}. Ce signal pourrait être attribué de façon erronée à une organisation moins compacte sur les couches les plus superficielles de la joue.

Dans le chapitre précédent, nous avons montré par micro-spectroscopie confocale Raman que le vieillissement cutané engendrait au niveau tissulaire un épaississement de la couche cornée. Cet épaississement associé à l'augmentation de la taille des cornéocytes peut en partie compenser la diminution significative de la teneur en lipides avec l'âge observée d'une part par spectrocopie infrarouge sur les couches les plus superficielles du SC de la joue et le site du bras exposé et d'autre part par chromatographie liquide ($I_{somme\ totale\ des\ aires\ des\ lipides}$/ $I_{aire\ de\ l'étalon\ interne}$). Il est également à noter que la teneur en sébum diminue avec l'âge, résultat observé par spectroscopie

infrarouge.

Au niveau supramoléculaire, les couches les plus superficielles du SC sont toujours organisées dans une phase orthorhombique en vieillissant mais cependant la fluidité du ciment intercornéocytaire diminue avec l'âge. La prévalence de la phase orthorhombique plus compacte corrèle avec une plus grande intégrité et efficacité de la fonction barrière cutanée.

L'efficacité de la fonction barrière dépend du maintien de la teneur en acides gras libres, en céramides et en cholestérol. Ainsi, nous nous sommes particulièrement intéressés à une potentielle variation des espèces céramidiques. En effet, cette famille lipidique a un impact majeur dans la qualité de la fonction barrière (Bouwstra et al. 1998; Di Nardo et al. 1998; de Jager et al. 2004). Le profil de l'ensemble de ces classes et sous-classes de céramides est obtenu en 24 min par NP-LC, (**Figure 8**) . La (**Figure 8**) représente les différents chromatogrammes obtenus: le chromatogramme général pour les sous-classes saturées en a, la sous-classe NH saturée en b), la sous-classe NP saturée en c) et la sous-classe AP saturée en d). Les chromatogrammes montrent la très grande hétérogénéité des sous-classes majoritaires. Il est difficile de les interpréter. Ainsi, nous avons décider de représenter sous forme de tableau les différents temps de rétention. Le couplage de la LC à la spectrométrie de masse haute résolution a permis d'observer 2230 composés parmi lesquels 85% ont été identifiés. L'ordre d'élution des classes de céramides dépend 1) principalement de la tête polaire des céramides et notamment du nombre de OH présent sur la tête polaire (dS, S, P, H et T), 2) du nombre d'insaturations de la chaîne alkyle et 3) la longueur totale des chaînes hydrocarbonées. La partie acide gras joue un rôle secondaire dans la rétention des céramides : céramides estérifiés, céramides non-hydroxylés, céramides hydroxylés et finalement les céramides liés à l'enveloppe cornifiée : [EO] < [(1-O-E)N] < [(1-O-E)A] < [N] < [A] < [O].

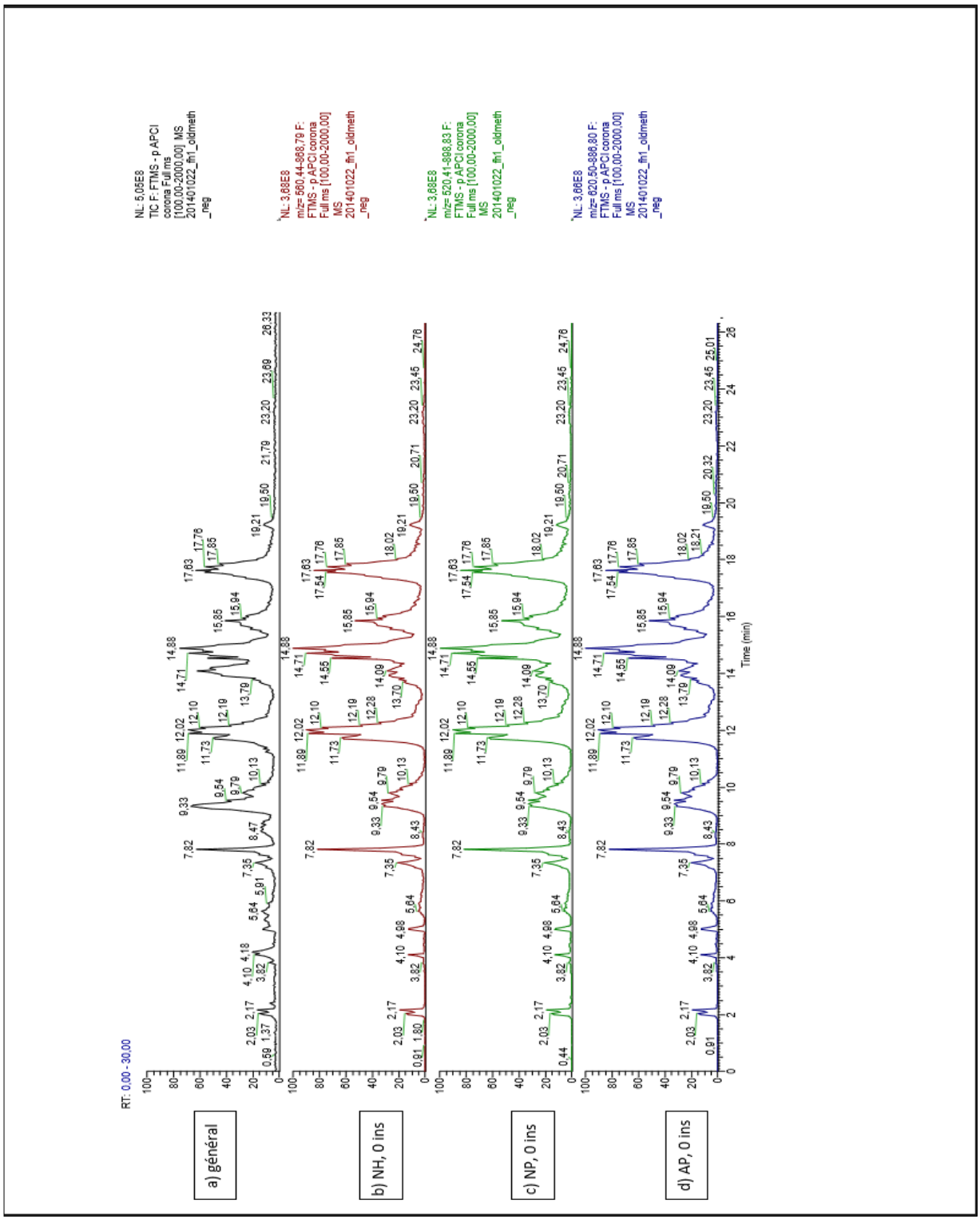


Figure 8: Chromatogrammes : *chromatogramme général en a), sous-classe NH saturée en b), sous-classe NP saturée en c) et sous-classe AP saturée en d.).*

Les deux classes de céramides les plus présentes dans le SC sont les H-CER et P-CER, (**Figure 9**). Globalement, la somme de ces deux classes de céramides est constante lors du vieillissement cutané. Les H-CER sont seulement trouvés dans l'épiderme humain et leur origine n'est pas complètement connue. Ils semblent provenir d'une enzyme hydroxylase qui hydroxyle les S-CER. Le rôle de cette classe de céramides n'est pas encore bien connu et compris même si les H-CER semblent essentiels au bon fonctionnement de la fonction barrière cutanée (Wakita et al. 1992). Le groupe hydroxyle supplémentaire peut renforcer le nombre et la force des liaisons H et conférer une plus grande cohésion de la matrice lipidique. Les P-CER peuvent être produits de deux façons différentes (Merrill 2002; Mizutani et al. 2009; Mullen et al. 2012; Rabionet et al. 2014) : le mode *de novo* qui consiste en la production des P-CER à partir des dS-CER sous l'action d'une enzyme appelée dihydrocéramide désaturase 2, et le mode direct par la sphinganine lors du *salvage pathway*, processus moins connu (Sandhoff 2010). Les P-CER semblent être responsables d'une organisation plutôt hexagonale (Rerek et al. 2001). Les dS-CER (environ 15%) et les S-CER (environ 10%) sont dans des proportions équivalentes dans le SC, quelque soit l'âge. L'action de la dihydrocéramide désaturase 1 sur la dihydrocéramide dS-CER produisant les S-CER n'est donc pas modifiée avec l'âge. Les S-CER induisent une organisation orthorhombique et une imbrication interlipidique très dense (van Smeden et al. 2014) impliqués dans la conservation de la fonction barrière avec l'âge. Les T-CER représentent moins de 5% des céramides dans les couches superficielles du SC.

Les sous-classes [N] sont majoritaires dans les S-CER, P-CER et H-CER. Les sous-classes [A] sont majoritaires dans les T-CER. Les trois sous-classes de céramides les plus importantes du SC sont CER(NH), CER(NP) et CER(AP). Ces résultats sont cohérents avec la littérature. La diminution de la teneur en CER(NP) contribue à la perméabilité anormale de la fonction barrière (Imokawa et al. 1991; Bleck et al. 1999; Macheleidt et al. 2002). Les CER(NH) augmentent avec l'âge. Le rapport CER(NH)/CER(NP) augmente avec l'âge, et de façon plus générale le rapport H-CER/P-CER. Une hypothèse serait que l'augmentation des H-CER avec l'âge augmenterait la cohésion des lipides lamellaires du

SC et compenserait ainsi la diminution des lipides du SC.

Les parties acides gras ω-estérifiées [EO] augmentent avec l'âge. La formation d'une Phase de Longue Périodicité, [2]LPP est observée avec la présence d'acylcéramides, [EO]. Les CER(EOS) (de Jager et al. 2004; Kessner et al. 2008) créent de l'espace dans le ciment intercornéocytaire qui est rempli par d'autres céramides à longue chaîne d'acide gras (Bouwstra et al. 1998) rendant ainsi la structure plus compacte. La formation de phase lamellaire LPP est toujours observée (de Jager et al. 2004; Kessner et al. 2008) en présence d'acylcéramides. La présence des CER(EOP) dans les structures est nécessaire pour créer de l'espace qui peut être rempli par des chaînes d'acides gras (Bouwstra et al. 1998) rendant la structure plus compacte (de Jager et al. 2004). Même en petite quantité, les CER(EOS) promeuvent la formation de LPP plus que les CER(EOP) (de Jager et al. 2004). La structure plus compacte diminue la fluidité du ciment intercornéocytaire (Bouwstra et al. 1998) contribuant ainsi à une meilleure fonction barrière avec l'âge. Dans le même temps, la sous-classe CER(EOH) augmente avec l'âge et comme la région de la tête polaire est plus grosse que celui des CER(EOS), cela rend le domaine plus fluide et donc plus perméable. On peut dire que bien qu'il soit nécessaire d'avoir un domaine très ordonné pour l'imperméabilité, il est également nécessaire d'avoir des parties moins compactes pour la flexibilité.

[2] *LPP : Long Periodicity Phase*

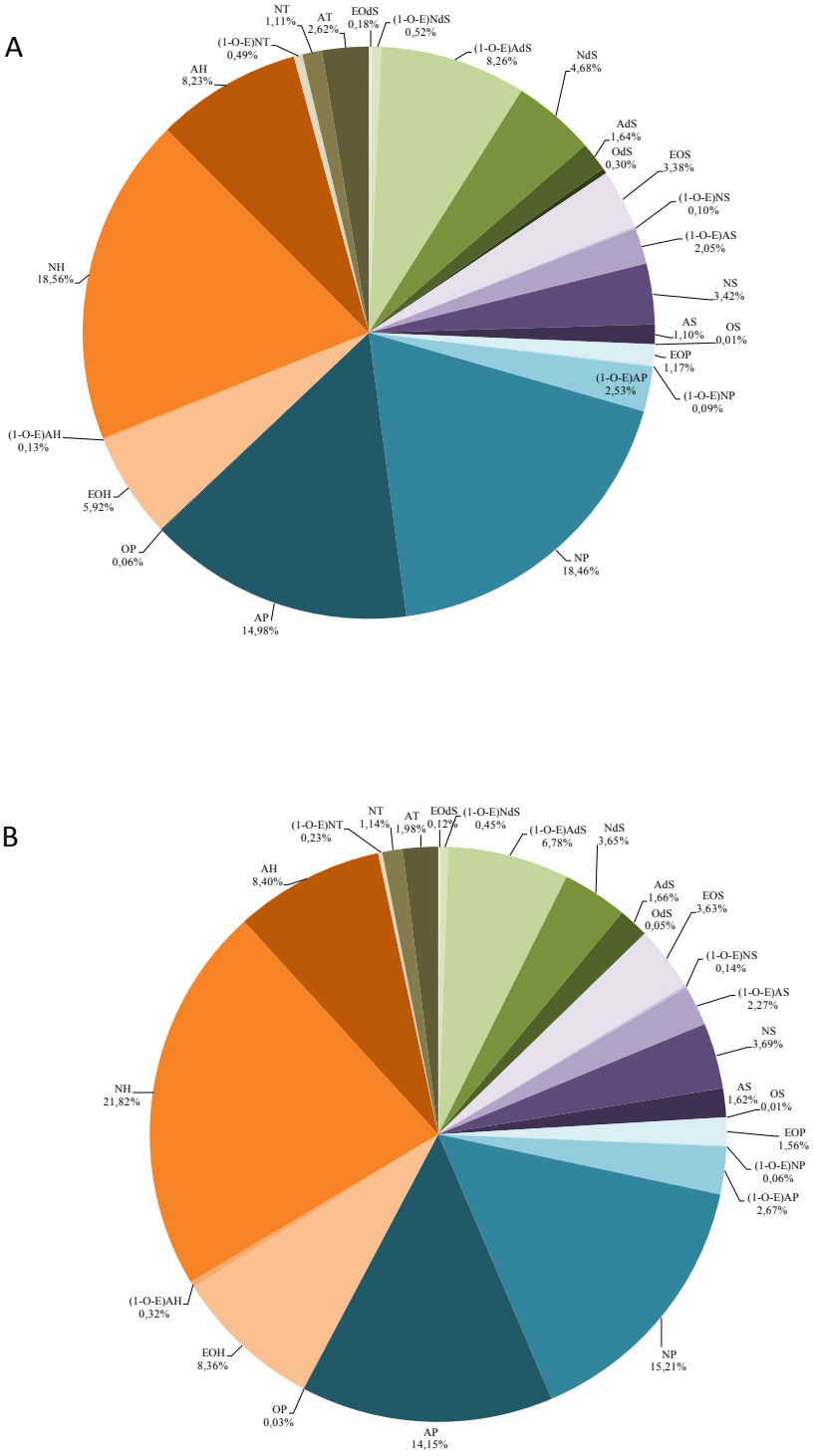


Figure 9 : Répartition pour chaque sous-classe de céramides pour les deux groupes d'âge*.Le diagramme montre la répartition en % de chaque sous-classe pour les deux groupes d'âge a) pour le SC des personnes jeunes et b) pour le SC des personnes âgées. Le pourcentage représente la quantité relative par rapport à la teneur totale des lipides du SC dans un groupe considéré.*

La longueur des chaînes des céramides augmente en vieillissant contribuant ainsi à l'amélioration de la fonction barrière. Les chaînes avec un nombre total pair de carbone sont plus abondantes que les chaînes avec un nombre total impair de carbone. Les céramides les plus abondants dans le SC des personnes jeunes sont constitués d'une longueur totale de chaîne de 42/44C et de 44/46C chez les personnes âgées (**Figure 10**). La longueur de la chaîne alkyle de la base sphingoide varie de façon limitée (C18- C22). La longueur de la chaîne d'acides gras (Rabionet et al. 2014) se définit selon trois tailles de céramides : [3]*Long Chain* (LC) avec 14 à 19C soit au total 32 à 43C, [4]*Very Long Chain* (VLC) avec 20 à 26C soit au total 38 à 50C et [5]*Ultra Long Chain*(ULC) >26C soit au total >50C. La partie acide gras ω-estérifié est obtenue par l'ajout d'une chaîne 18C provenant de l'acide linoléique. La distribution est tri-modale quelque soit le nombre d'insaturations de la chaîne carbonée. L'intensité du signal diminue d'un facteur 5 entre la distribution tri-modale des chaînes totales saturées et celle des chaînes totales monoinsaturées. La distribution des chaînes carbonées saturées et monoinsaturées dépend de la longueur totale de la chaîne. La classe ayant le plus grand signal pour les céramides à chaîne saturée vient des H-CER mais ceux-ci ne sont pas les plus nombreux. Les céramides les plus nombreux sont ceux qui portent une partie acide gras α-hydroxylé, [A], indépendamment de la tête polaire. En ce qui concerne les ULC pour toutes les chaînes de céramides saturées et monoinsaturées, les céramides avec une chaîne d'acide gras estérifiée avec un groupe hydroxyle sur la base sphingoide [(1-O-E)A] sont les plus nombreux. L'intensité des signaux diminue d'un facteur 10 entre la distribution tri-modale des céramides monoinsaturés et celle des céramides diinsaturés. Toutes les longueurs totales de chaînes ne sont pas représentées et les céramides avec une chaîne ULC sont majoritaires. Les céramides diinsaturés sont principalement des

[3] Longue chaîne

[4] Très Longue Chaîne

[5] Ultra Longue Chaîne

céramides ω-estérifiées [EO] avec une tête polaire P. Le signal majoritaire des ULC pour les chaînes de céramides diinsaturées vient des céramides ω-estérifiés [EO] mais les céramides avec une chaîne d'acide gras estérifiée avec un groupe hydroxyle sur la base sphingoide [(1-O-E)A] sont les plus nombreux. Ces céramides ULC jouent un rôle important dans le vieillissement cutané et sont nécessaires à une perméabilité normale de la barrière (Feingold and Elias 2014).

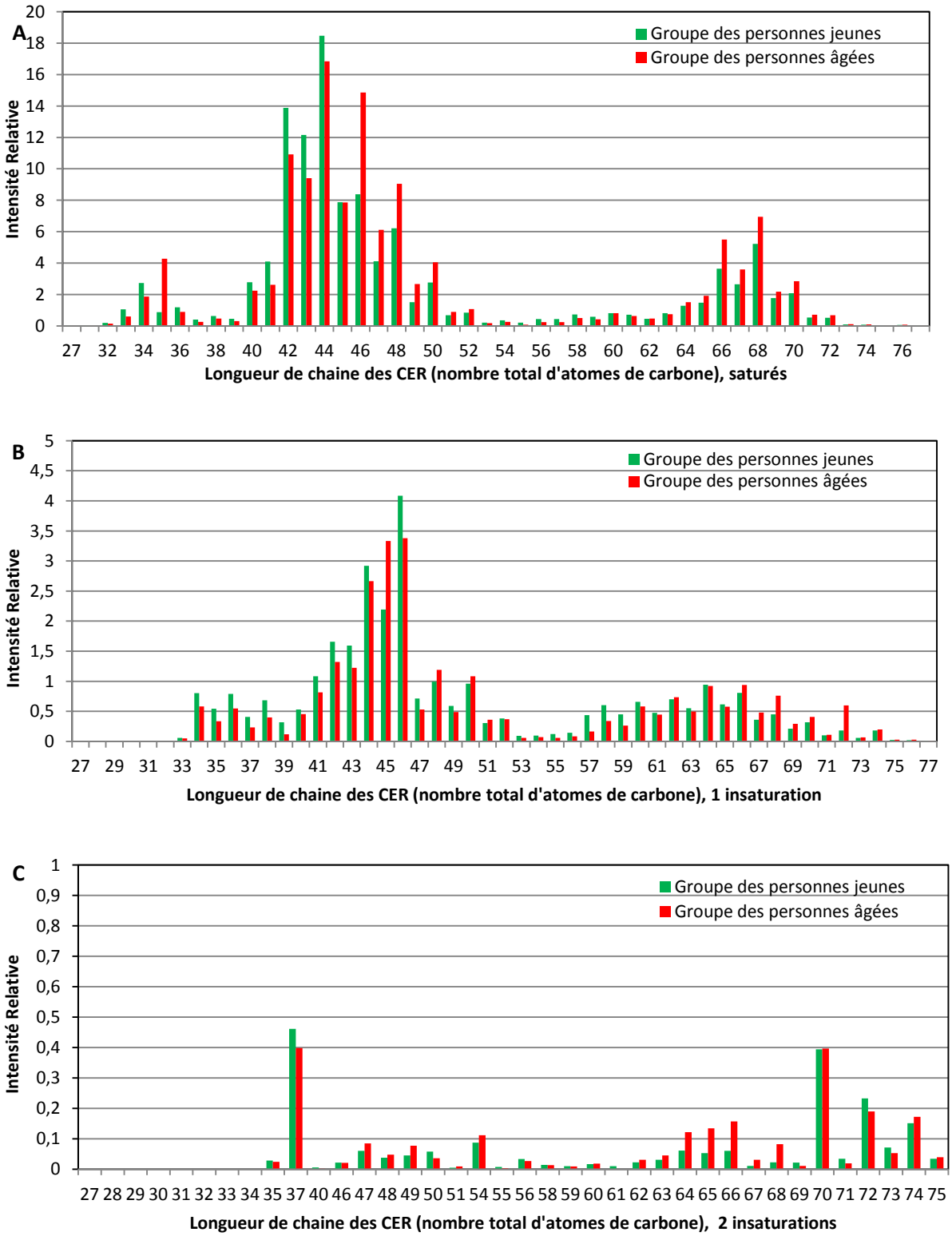


Figure 10 **: *Diagrammes en*** *barre représentant la longueur totale de la chaine carbonée en fonction de l'intensité relative pour les céramides saturés A), monoinsaturés B) et diinsaturés C).*

Les espèces moléculaires différentes entre les deux groupes d'âge sont à peu près de 40 espèces dont 75% sont présentes chez les personnes jeunes et 25% chez les personnes âgées. Les différences inter-groupe sont les plus observées pour les classes T-CER et dS-CER. Plus précisément pour les dS, 1-O-acylcéramides (3 chaînes alkyles) avec une chaîne d'acide gras α-hydroxylé sont les sous-classes les plus différentes. La classe H-CER ne présente aucune différence intergroupe.

4 | Conclusion

L'âge n'est pas un facteur critique pour la teneur en lipides et l'organisation au niveau des couches supérieures de la SC. Le SC des personnes âgées contient moins de lipides, et en particulier moins de céramides. Ces diminutions sont compensées par l'augmentation de l'épaisseur de SC avec l'âge dûe au ralentissement du taux de desquamation. Il est intéressant de constater que quelque soit l'âge, les lipides du SC sont organisés dans une phase orthorhombique. Seules quelques variations sont observées avec l'âge liées à de légères modifications de la micro-hétérogénéité des céramides dans la matrice lipidique, mais ces variations ne représentent que 2% des espèces moléculaires. La compacité des lipides du SC est légèrement plus élevée avec l'âge probablement du fait de l'augmentation de la longueur des chaînes carbonées et l'augmentation du rapport H-CER/P-CER. Cette augmentation de la compacité des lipides conduit à une diminution de la fluidité du ciment intercornéocytaire avec l'âge. L'ensemble de ces résultats maintient la fonction barrière cutanée avec l'âge chez les peaux saines.

IV ARTICLE 2

Chronological Aging Impact on the Skin Barrier Function: the Stratum Corneum Lipid Composition and Molecular Organization

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ABSTRACT

Objective: Lipids are an important contributor to the skin barrier function. The objective of this study was to evaluate potential effects of age and body site location on the composition of the *Stratum Corneum* (SC) lipids. **Methods:** The study was conducted on 20 healthy women volunteers in a temperature and humidity controlled room. They were divided into 2 groups according to age (younger: 20-30 and older: 55+ years). SC lipid composition and organization were examined using Attenuated Total Reflectance – Fourier Transform Infrared (ATR-FTIR) spectroscopy and lipid extraction followed by analysis by reversed-phase liquid chromatography - high resolution mass spectrometry (LC-HRMS). The measured sites included the face (center of the cheek) and on two sites on the arm: one relatively protected (upper inner arm) and one exposed (dorsal arm). **Results:** IR data showed that the lipid/protein ratio slightly decreased with age on the three skin sites. Also independent of age, the relative values of lipid/protein ratio for the three sites were higher on face > protected arm > exposed arm. Moreover the lipid compactness is conserved with age on the three skin sites of interest. LC-HRMS data showed that, although relative amounts of lipid classes are not significantly modified, some qualitative modifications within ceramides occurred with aging. **Conclusions:** SC lipid composition appears to be only slightly dependent on age in a site-dependent manner and consequently mildly impact the SC lipid organization.

Keywords: skin lipids, ATR-FTIR, LC-HRMS, ceramides

INTRODUCTION

The Stratum Corneum (SC) protects the human skin from desiccation and pathogen invasion. It is composed of corneocytes embedded in a lipid matrix. This lipid matrix is produced inside the cells and the secreted during the differentiation of kératinocytes into corneocytes as they migrate towards the skin surface. The keratinocytes in the Stratum Granulosum (SG) contain a high number of membrane-coating granules referred to as the Lamellar Bodies (LB), in which lipids are stored: sphingomyelin, phospholipids, and glucosylceramides. The stored lipids in the LBs are released together with hydrolytic enzymes into the intercellular space at the SG/SC interface in a process that is called exocytosis (1–3). The intercellular lipids are composed of ceramides (CER), free fatty acids (FFAs), and cholesterol in an approximately equimolar ratio (4–8). To maintain an efficient SC barrier function it is important to have a balance between these three components (9). Other minor components in the lipid matrix such as cholesterol sulfate play an important role in barrier homeostasis. Lipids are implicated as an important determinant of the water-retaining properties of the SC by forming a multilamellar architecture within intercellular spaces between SC cells (10–12). In human skin the extracellular lipids are arranged in a characteristic molecular (lateral and lamellar) organization to confer a low water permeability to the SC:

1) lamellar organization: they form two coexisting crystalline lamellar phases: the Long Periodicity Phase (LPP) and the Short Periodicity Phase (SPP) with characteristic distances of 13 nm and 6 nm respectively (13–18).

2) lateral organization: at normal skin temperature, the hydrocarbon lipid chains in these lamellae form an orthorhombic packing (ORT) with a small fraction of the lipids forming hexagonal (HEX) and liquid-crystalline (LIQ) structures (13–16).

The quality of the skin barrier function is globally conserved with aging (17). However the chronological aging and the photoaging impact the structure and the composition

of the SC and particularly they impact the lipid matrix that plays a major role in the lipid skin barrier function (18). As the lipid matrix majoritary contains ceramides that play a major role in the barrier function the molecular signature of the ceramides has been investigated. The aim of this study has been the investigation of aging on SC lipid composition and organization and skin barrier function.

MATERIAL AND METHODS

Reagents

Heptane and methanol were obtained from Sigma-Aldrich (St. Louis, MO). For High Pressure Liquid Chromatography (HPLC): chloroform was obtained from VWR (Dublin, Ireland), ethanol, isopropyl alcohol, methanol, and n-heptane of HPLC grade or higher were purchased from Fisher Scientific (Fisher Scientific, Pittsburgh, PA). The omega 6 ceramide®, ceramide-like, internal standard, has been kindly furnished by Solabia (Pantin, France) and is an amino propan-diester coming from the palmytic acid and linoleic acid.

Subjects

The study was conducted in accordance with the ethical principles of The Declaration of Helsinki. Healthy female volunteers of Fitzpatrick skin types I-III with no dermatological problems participated in the study following signed informed consent. They were divided into 2 groups of 10 based on their age: G1: 18–30 and G2: 65–75 years of age. All measurements were performed following 15 min of acclimatization in an environmentally controlled room (20–25°C, 40% relative humidity). They accepted not to expose themselves intentionally for a long time to the sun (or tanning beds) for at least 1 month before measurement and not to use self-tanning products, which might interfere with the Raman measurements by introducing high background fluorescence. They were not allowed to use any skin care product or deodorant or

have a warm drink the morning of the test. Non-invasive measurements were performed on three skin body sites: face (central cheek area), relatively exposed arm site (dorsal forearm), and relatively protected arm site (upper inner arm).

ATR-FTIR spectroscopy

ATR-FTIR was used to collect high quality IR spectra of the most superficial layer of the SC (2µm). Spectra were recorded using an FTIR spectrometer (Nicolet™ 6700, Thermo, Madison, WI, USA) equipped with a Horizontal Attenuated Reflectance accessory (HATR Plus™, Pike Technologies, Madison, WI, USA), a flat plate ZnSe crystal, and a DTGS KBr, deuterated triglycine sulfate detector, and a KBr beam splitter for mid-IR 4000–650 cm^{-1}. Spectra were generated from 32 scans operating with an optical resolution of 4 cm^{-1}. ATR-FTIR spectroscopy data were processed with OMNIC™ 7.0 software (Thermo Electron, OH, USA) and data plots were generated using Excel (Microsoft Office, 2007, WA, USA). Before each measurement a blank reference spectrum was collected. The volunteers placed the skin site of interest against an ATR crystal to collect ATR-FTIR *in vivo* spectra of the skin. Three spectra were recorded at each site.

SC lipid bands between 2800 and 3000 cm^{-1} are due to C-CH stretching vibrations associated with the lipid alkyl chains (19). The lipid content on the skin surface is evaluated by the ratio of the CH stretching vibration band of lipids between 2879- 2946 cm^{-1}/ C-H stretching vibration band of protein between 2946-2975 cm^{-1}. The absorbance near 2850 cm^{-1} is related to the symmetric v_s (CH_2) vibrations. The frequency of this band is sensitive to the lateral organization of the alkyl chains. The triglyceride content on the skin surface can be assessed by the C=0 stretching band of lipid ester carbonyl and indicates the presence of sebum at the skin surface. All spectral information are summarized in **Table 1.**

TABLE 1. Spectral information *used to analyze the spectra in confocal Raman microspectroscopy and ATR-FTIR spectroscopy. The lipid content is evaluated by the ratio of the C-H stretching vibration band of lipids / C-H stretching vibration band of protein, the lipid organization at the skin surface is evaluated by the shift of the vibrational symmetric v_s CH_2 band and the sebum content on the skin surface is evaluated by measuring the C=0 stretching band of lipid ester carbonyl.*

Information	**Spectral analysis**	**Bands**	**Ref**
Lipid/protein	Intensity of the CH stretching vibration band of lipids	2879-2946 cm^{-1}	Kollias& Stamatas(20)
	Intensity of the CH stretching vibration band of protein	2946-2975 cm^{-1}	Kollias& Stamatas(20)
Lipid organization	Shift of vibrational symmetric v_s CH_2 band	2848 cm^{-1}	Moore(21)
Triglycerides	Intensity of the C=0 stretching band of lipid ester carbonyl	1740 cm^{-1}	Brancaleon(22)

SC Lipid collection

Cotton swabs were washed in a solution of (Chloroform: Methanol, 2:1) and let to dry overnight. They were used to remove the SC lipids at the skin surface according to the following protocol: Four cotton swabs were used on one site (protected arm site) to collect the SC lipids at the skin surface. Each swab was placed in a vial and stored in a freezer at -20°C until further use. The collected lipids were extracted with a mix of (Chloroform: Methanol, 2:1) containing the internal standard and then transferred into a 2mL-vial and dried under a gentle stream of nitrogen. Then, the residues are re-dissolved with 250 µL of a solution of (Chloroform: Heptane, 1:9).

Ceramide analysis by LC/MS

All samples from the lipid collection were analyzed using a single setup of HPLC (Ultimate 3000 Dionex, Thermofisher Scientific, San Jose, CA). Separation of epidermal lipids can be achieved on a polyvinyl alcohol (PVA)-Sil normal phase column (PVA-bonded column; 5 µm particle size, 100 × 2.1 mm i.d.) purchased from YMC (Kyoto, Japan). HPLC was performed using a gradient solvent system of heptane/chloroform, 80/20 (solvent A), acetone (solvent B) and isopropyl alcohol (solvent C) using a flow rate of 1 ml/min from 100% A to 50% of B in A in 25 min with an additional 3 min of column washing with isopropanol and 10 min equilibration afterwards, leading to a total run time of 40 min.

The HPLC was coupled to a mass spectrometer equipped with an Atmospheric Pressure Chemical Ionization (APCI) probe and an hybrid mass spectrometer LTQ-Orbitrap Velos Pro (Thermofisher Scientific, San Jose, CA) operating in a full scan and Higher Collision Dissociation (HCD) fragmentation in data dependant mode on the top 5 ions to profile skin ceramides. Using APCI-MS different ceramides classes can be most effectively analyzed in negative ion mode. The vaporization temperature of the source heater was set to 400°C and the heated capillary was set to 350°C. The capillary voltage was maintained at 6 kV and the discharge current was set to 4 µA. The flow rates of the nitrogen sheath and auxiliary gas were set to 40 and 5 l/min, respectively. To obtain a full CER profile including all CER chain lengths, the scan range was set from 450–750 atomic mass units (amu) and 750-2000 atomic mass units (amu). Fragmentation was performed in HCD mode with 30 eV of collision energy. The resolution in full scan was set to 100000. The injection volume of all samples was set to 10 µl. The analysis was performed using Thermo Fischer Xcalibur software (version 2.0).

CER Nomenclature

The literature used to report 15 CER subclasses present in the extracellular space of the human SC (5, 23, 24). A ceramide molecule is composed of a fatty acid (FA), linked by

an amide bond to a sphingoid base moiety. In the SC five different types of sphingoid bases have been recently inventoried (25):

- S: Sphingosine
- dS: dihydroxysphingosine or sphinganine
- P: Phytosphingosine
- H: 6-hydroxy-sphingosine
- T: dihydroxysphinganine (26)

There are five types of fatty acid moieties in CER:

- N: Non-Hydroxylated-/non esterifiedfatty acid chain
- A: α-hydroxylated fatty acid chain: contains a hydroxyl group
- EO: ω-esterified fatty acid chain: contains linoleic acid
- O: ω-hydroxylated fatty acid moiety linked to the cornified envelope of corneocytes(27)
- 1-O-E: contains one more fatty acid moiety, esterified with the primary OH group in the sphingoid base(23)

The nomenclature is based on the molecular structure of the ceramides. Motta et al. (28) and then modified by Robson et al. (25) designed the ceramides class by a minimum of 2 letters. The first letter indicates the type of fatty acid attached in N-linkage (N, A, EO, O or (1-O-E)). The last letter defines the sphingoid base (S, dS, P, H or T), see in **Table 2**. For example, CER(NS) 50:0, corresponds to the ceramide with a sphingosine base linked to a Non-Hydroxylated saturated fatty acid chain with 50 carbons and 0 unsaturated bonds.

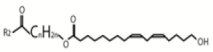
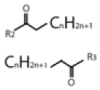
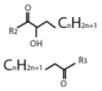
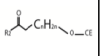
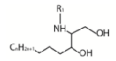
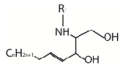
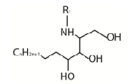
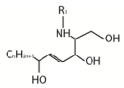
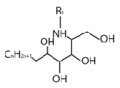
	 N	 A	 EO	 (1-O-E)N	 (1-O-E)A	 O
 dS	CER(NdS)	CER(AdS)	CER(EOdS)	CER((1-O-E)NdS)	CER((1-O-E)AdS)	CER(OdS)
 S	CER(NS)	CER(AS)	CER(EOS)	CER((1-O-E)NS)	CER((1-O-E)AS)	CER(OS)
 P	CER(NP)	CER(AP)	CER(EOP)	CER((1-O-E)NP)	CER((1-O-E)AP)	CER(OP)
 H	CER(NH)	CER(AH)	CER(EOH)	CER((1-O-E)NH)	CER((1-O-E)AH)	CER(OH)
 T	CER(NT)	CER(AT)	CER(EOT)	CER((1-O-E)NT)	CER((1-O-E)AT)	CER(OT)

***TABLE 2. Nomenclature and structure of ceramides**. All CERs are constituted of a polar head group and two or three alkyl chains. Each CER subclass is defined by its sphingoid base and fatty acid chain. 30 CER subclasses are represented. Abbreviations for the sphingoid base: dS, dihydrosphingosine; S, sphingosine; P, phytosphingosine; H, 6-hydroxy sphingosine; T, dihydroxysphinganine Abbreviations for the acyl chain: N, Non-Hydroxylated-/ non esterified-fatty acid chain; A, α-hydroxylated fatty acid chain; EO, ω-esterified fatty acid chain; 1-O-E, contains one more fatty acid moiety, esterified with the primary OH group in the sphingoid base; O, ω-hydroxylated fatty acid moiety linked to the cornified envelope.*

Statistical analysis of spectral data

Statistical comparison of the spectral data of the two age groups and of three skin sites were performed using the appropriate t-test following the Anderson-Darling normality test and test of variance (F-test). Statistical significance was accepted at the level of $\alpha=0.05$.

All the abbreviations are summarized in the **Table 3.**

ATR-FTIR	Horizontal Attenuated Total Reflectance - Fourier Transform Infrared spectroscopy
DTGS KBr	Deuterated triglycine sulfate detector potassium bromide
LC-HRMS	Liquid chromatography - high resolution mass spectrometry
RP-LC	Reverse Phase Liquid Chromatography
NP-LC	Normal Phase Liquid Chromatography
PVA	Polyvinylalcohol
ORT	Orthorhombic packing
LIQ	Liquid phase
HEX	Hexagonal packing
dS	dihydrosphingosine
S	Sphingosine
P	Phytosphingosine
H	6-hydroxy-sphingosine
T	dihydroxysphinganine
N	Non-Hydroxylated-/ non esterified- fatty acid chain
A	α-hydroxylated fatty acid chain
EO	ω-esterified fatty acid chain
O	ω-hydroxylated fatty acid moiety
1-O-E	1-O-Acyl ceramides
dS-CER	Ceramide with a dS base
S-CER	Ceramide with a S base
P-CER	Ceramide with a P base
H-CER	Ceramide with a H base
T-CER	Ceramide with a T base
ULC	Ultra Long Chain
VLC	Very Long Chain
LC	Long Chain

*TABLE3. **Abbreviations** used are reported.*

RESULTS

To study the effects of age on the SC lipids we studied the lipid content and the lateral organization as well as the molecular composition.

SC lipid content and lateral organization are slightly affected by age and differ between sites

The obtained spectroscopic results are summarized in the **Fig.1**. The obtained results are very homogenous within a group with a very weak inter individual variability as indicated by the small standard deviations.

The intercellular lipid content is expressed as lipid-to-protein ratio, evaluated by $\nu_{sCH\ lipid,\ (2879\text{-}2946cm\text{-}1)}/\nu_{s\ CH\ protein,\ (2946\text{-}2975cm\text{-}1)}$. The results show that regardless of the group of age, the site on the face contains more SC lipids than on the two arm sites. The protected arm site contains more SC lipids compared to the exposed arm site. The results in the uppermost layers of the SC of the face compared to the two skin sites are in accordance with those found by Boireau et al. using microspectroscopy confocale Raman in the deep SC (17). With aging the SC lipid content decreases on the three skin sites and the decrease is higher on the face and the exposed arm site ($p<0.05$).

The evolution of the lateral packing with aging is observed by investigating the shift of symmetric vibrational band $\nu_s\ CH_2$ in the range 2848-2849.5 cm^{-1}. The SC lipids of the three skin sites of interest assume a more orthorhombic phase independent of age. However the packing fluidity decreases on the three skin sites as the $\nu_s\ CH_2$ decreases with aging. Regardless of the age, no statistical difference in the organization of the SC lipids was found between the two arm sites. The lateral organization in the uppermost layer of the SC on the face was found to be more orthorhombic than on the two arm sites ($\nu_s\ CH_2$ face > $\nu_s\ CH_2$ arm sites).

The sebum content is characterized by the intensity of the triglycerides C=0 stretching band of lipid ester carbonyl around 1740 cm^{-1}. On the face the sebum content was found to be higher than on the two arm sites for both age groups ($I_{1740cm\text{-}1,\ face} > I_{1740cm\text{-}1,}$

arms). The sebum content decreases on the three skin sites of interest with aging ($p<0.05$).

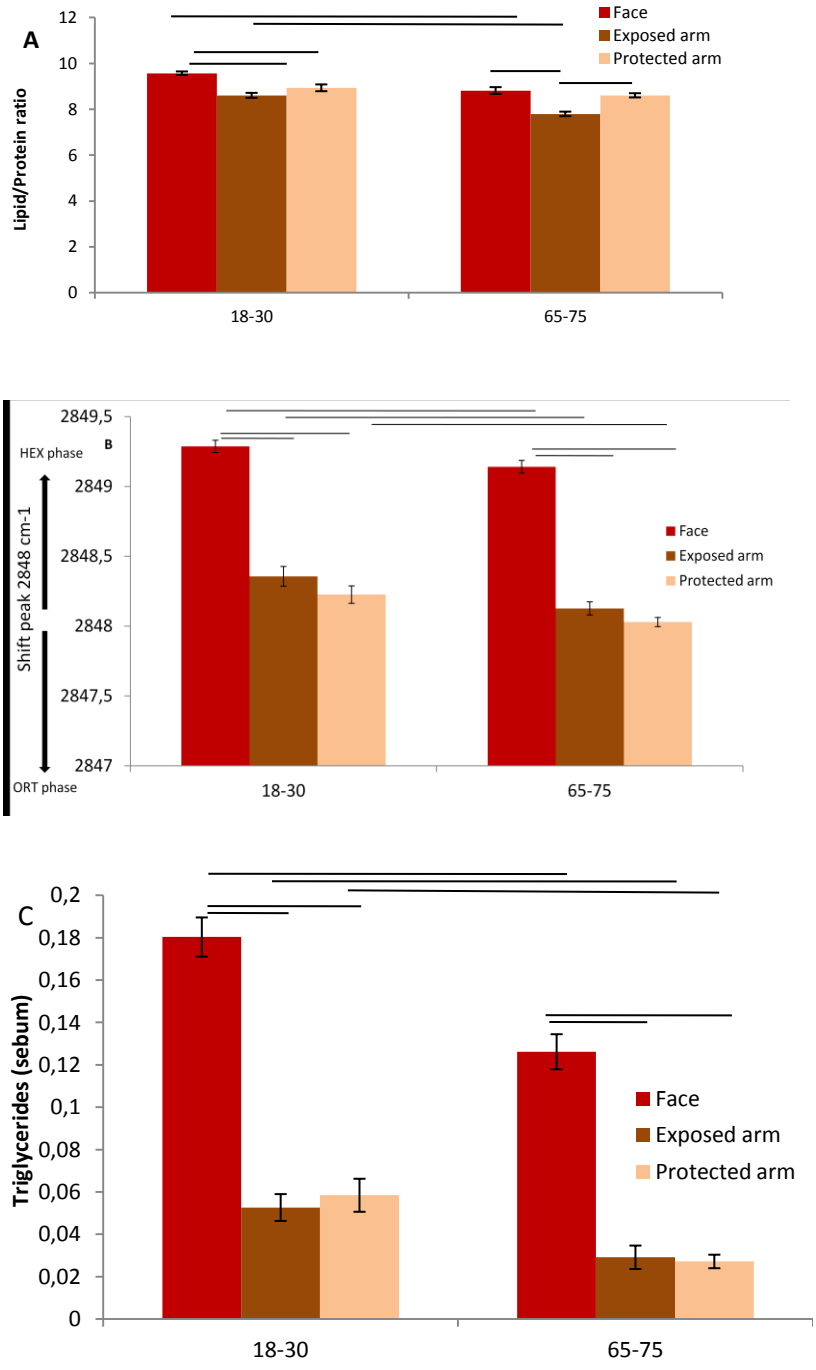


Fig.1 The SC lipid content and organization and sebum content are representated for the young and old groups*. a) SC lipid content in the uppermost layers is evaluated for 18-30 and 65-75 years old, b) the supramolecular organization of the SC in the uppermost layers is*

evaluated for 18-30 and 65-75 years old, c) the sebum content at the skin surface is evaluated for 18-30 and 65-75 years old.

Ceramide classes repartition as a function of age

Elution order of the ceramide classes

The SC is composed of CER, FA, and cholesterol (12, 29–33). The proportions of the three components are conserved with aging (34, 35). The ceramides are divided into subclasses which are major components for a proper barrier function (36–39) which is why we focus on the ceramide species.

The FA moiety also varies in chain length and in degrees of saturation (40) and give some micro-heterogeneity within each subclass.

Profiling of the SC CER classes has been obtained by Normal Phase-HPLC. The identification of the ceramides components has been done with an APCI/HR-MS detector, in negative mode leading to the $[M+Cl]^-$ adducts occurring in an isotopic massif. The most abundant peak is selected to identify the compounds of interest. The direct metabolomic approach is difficult because of presence of the chlorine isotopic massifs (Cl35 or Cl37) that adds peaks. That is why we realized an identification approach from the most abundant peak of each isotopic massif. Thus each chromatogram is transposed into a quantitative matrix.

By coupling LC to HRMS about 2230 compounds were observed. Among them, 1411 compounds have been identified representing 85.1% of the total SC lipid composition. Overall the chromatographic results are presented in the **Fig.2** and give qualitative information of the elution of the identified with CER class, CER subclasses, number of carbon atom and number of lipid chain unsaturations.

The chromatogram shows an elution order mainly according to the polarity of the head group of the CER classes with a broadening of chromatographic peak due to the structural micro-heterogeneity. Within each CER class, overlapping of some subclasses

exists with the high structural micro-heterogeneity of the FA moieties.

According to the chromatogram, the elution ranges of the subclasses are different: dS< S < P < H < T. The retention range depends on 1) the number of OH on the polar head group (the more OH groups on the polar head, the higher the retention) 2) the number of unsaturations of the alkyl chain (the more saturated the alkyl chain is, the lowest the retention) and 3) the total carbon chain length of the CER. However the carbon chain length has a small impact on the polarity of the species and thus changing the retention time slightly. As an example, at 5.3 min CER(EOdS) 72:0 and at 5.9 min CER(EOdS) 54:0.

Nevertheless, the FA moiety also plays a secondary role in retention. Within each CER classes, the elution of the subclasses are ordered as: [EO] < [(1-O-E)N] < [(1-O-E)A] < [N] < [A] < [O].

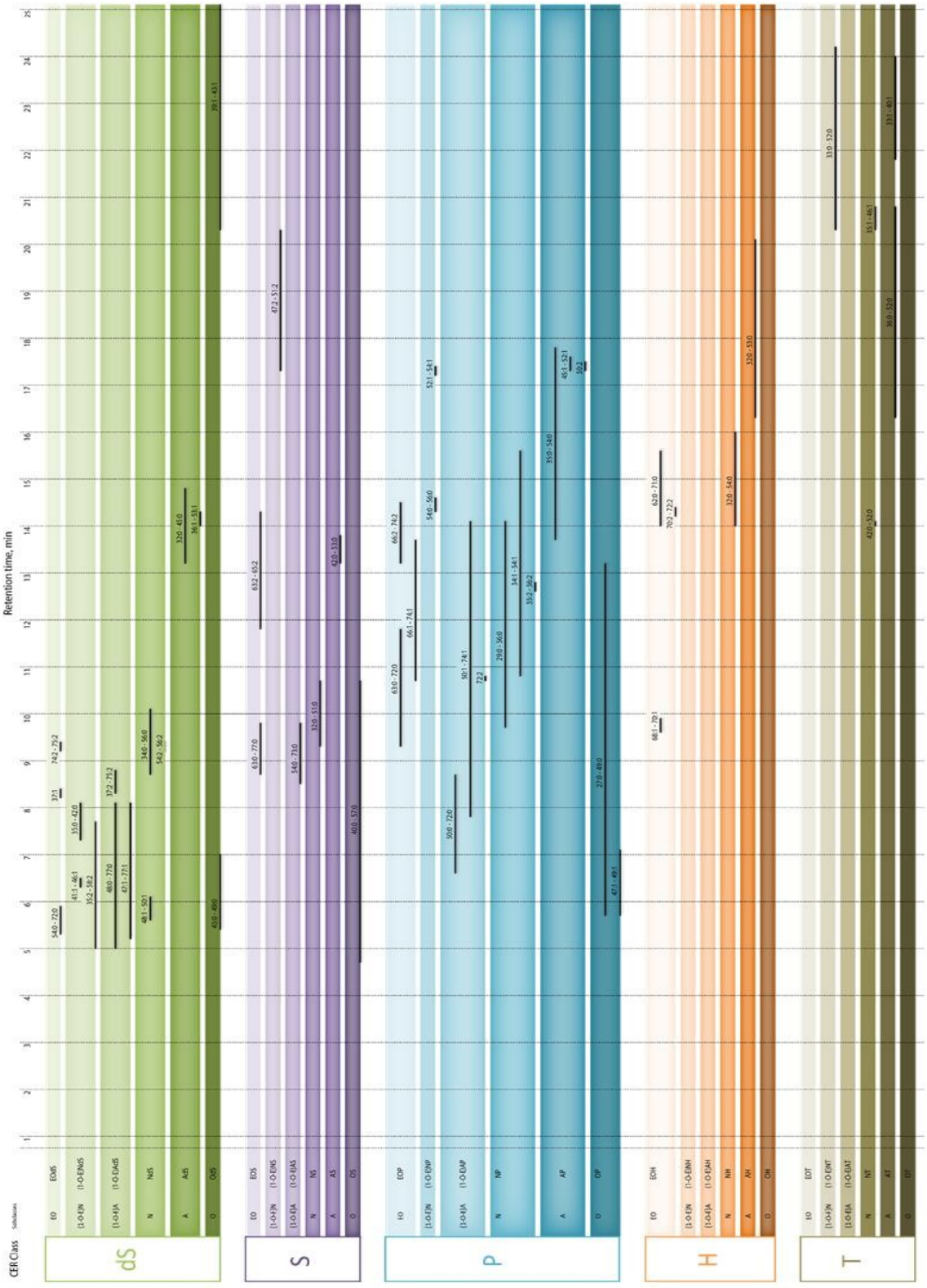


Fig. 2. Retention time *is given for ceramide classes and subclasses with the total number of carbons and insaturation numbers. The graph shows the elution of all ceramides classes and subclasses in function of the polar head group. Not all the 30 subclasses of ceramides (described in* ***Table 1****) were found.*

Repartition by CER classes and subclasses

The semi-quantitative repartition of the SC lipids into different subclasses and subclasses are based on internal normalization. The repartitions of the different subclasses are represented in **Fig. 3.** In each age group the most abundant ceramide classes are H-CER (about 33% for the young group and about 39% for the old group), and P-CER (about 37% for the young group and about 34% for the old group). Globally the sum of the percentages of H-CER and P-CER is similar for both age groups and represent more than half of the total ceramides. The percentages of S-CER (about 10%), dS-CER (about 15%), and T-CER (about 3%) are similar for the two age groups.

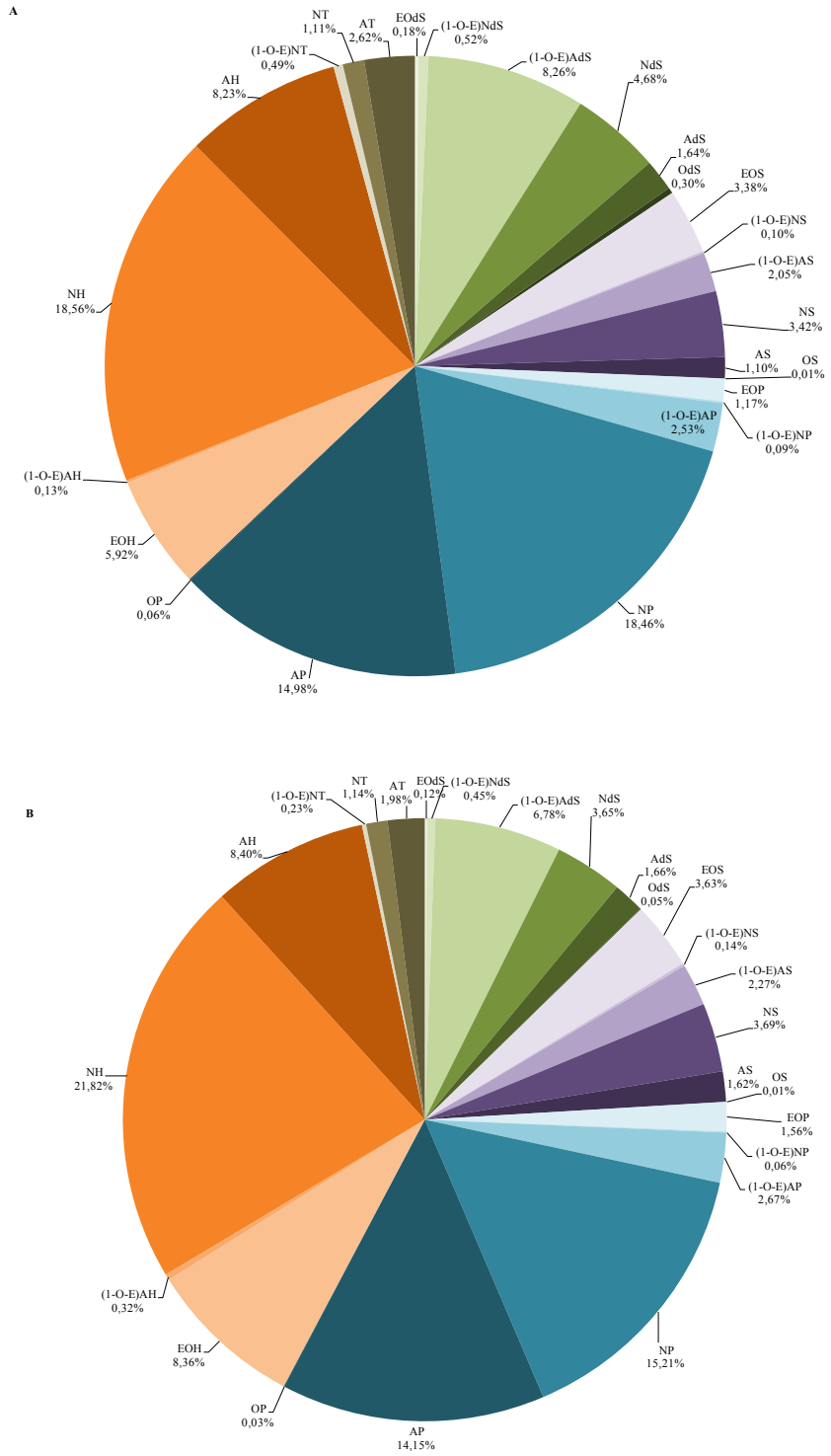


Fig.3. *The diagram shows the repartition in % of each subclass for the two age groups a) for the SC of the younger group and b) for the SC of the older group. The percentage represents the relative quantity compared to the total SC lipid content in one considered group.*

The radar diagram in **Fig.4** shows the relative repartition according to the ceramides classes: dS, S, P, H and T. Major CER subclasses are differently present within each CER class: non-hydroxylated-/ non-esterified [N] subclasses are major in S-CER, P-CER and H-CER. Hydoxylated [A] subclass is major in T-CER. The minority subclass is globally ω-hydroxylated fatty acid linked to the cornified envelop [O]: the higher percentage is found with dS base (about 0.4%) since the more numerous compounds (wider micro-heterogeneity) are observed in S-CER and P-CER. About 90% of hydroxylated-/esterified- CER ([(1-O-E)A]) belong to dS-CER and S-CER. The ω-esterified [EO] CER are major for the S-ceramides and the H-ceramide class. As they confer an orthorhombic organization, (37) they are essential for the skin barrier function.

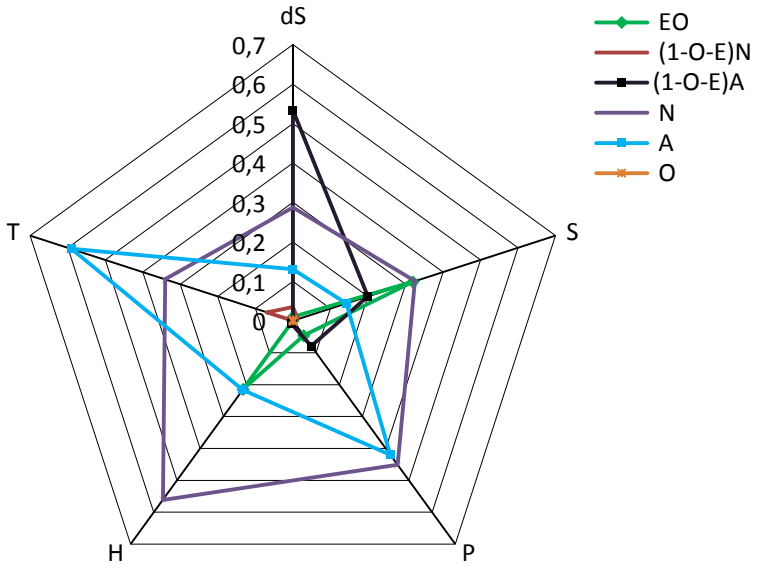


Fig.4 *The radar diagram shows the representation of the fatty acid moiety in function of the polar head group.*

Distribution of SC lipids according to the total number of carbons and number of unsaturations

The distribution of the carbon chain length is trimodal according to the three types of

carbon chain length, **Fig.5** (23): Long Chain C14-C19 (LC), Very Long Chain C20-C26 (VLC), Ultra Long Chain <C26 (ULC). To these FA chains, one C18 chain (from linoleic acid) is added in esterified ω-hydroxy fatty acid moiety, O. The distribution is observed with the fatty acid moieties that are saturated and monounsaturated. The trimodal distribution does not exist anymore with the diunsaturated fatty acid moieties. The saturated lipid chains are 5 fold more expressed than the monounsaturated ones, themselves 40 fold more expressed than diunsaturated lipids. The carbon chain length of the ceramides is conserved regardless the number of unsaturations.

The trimodal distribution observed for the saturated and monounsaturated CER may be linked to the definition of chain length of fatty acids with LC C14-C19, VLC C20-C26 (VLC), ULC <C26 (ULC)(23). With a C18-C20 sphingoid base, a LC of fatty acids corresponds to a total carbon chain length of ceramides between 32-43 carbons, a VLC of FA corresponds to a chain length of ceramides between 38-50 carbons, and a ULC of FA corresponds to a chain length of ceramides superior 50 carbons. Moreover the **Table 4** shows that the contribution of the VLC ceramides is higher for the saturated and the monounsaturated in each age group whereas the ULC ceramides contributes more in the diunsaturated ceramides. The **Table 4** also shows that the ULC ceramides are more abundant in aging and are required for a normal permeability barrier (41).

Concerning the diunsaturated ceramides (ω-esterified ceramides containing a linoleic acid moiety and mainly CER(EOP)), not all chain lengths are represented and the trimodal distribution is not well defined compared to the saturated and monounsaturated ceramides.

In the **Table 5** are the major signals found and the most numerous subclasses in each definition of chain length in saturated, monounsaturated, and diunsaturated ceramides. The main contribution of saturated ceramides comes from the H-ceramides but they are not the most numerous. The α-hydroxylated fatty acids are the most abundant in the monounsaturated ceramides subclasses. The diunsaturated ceramides, characterized by the long chain length with more than 70 C, are mainly ω-esterified P-

ceramides. Among the ULC ceramides, the ω-esterified CER, EO, are the most abundant as the acylceramides, (1-O-E), are the most numerous.

It should be noticed that in each part of the trimodal distribution within the VLC and ULC, on the left side of the median (lower total carbon atoms) the ceramide amount in the younger group is higher than that in the older group. On the right side of the median (higher total carbon atoms), the inverse situation is observed. This increase of the carbon chain length contributes to improving the skin barrier function as Janssens et al reported: the TEWL decreases proportionally with increasing chain length (42).

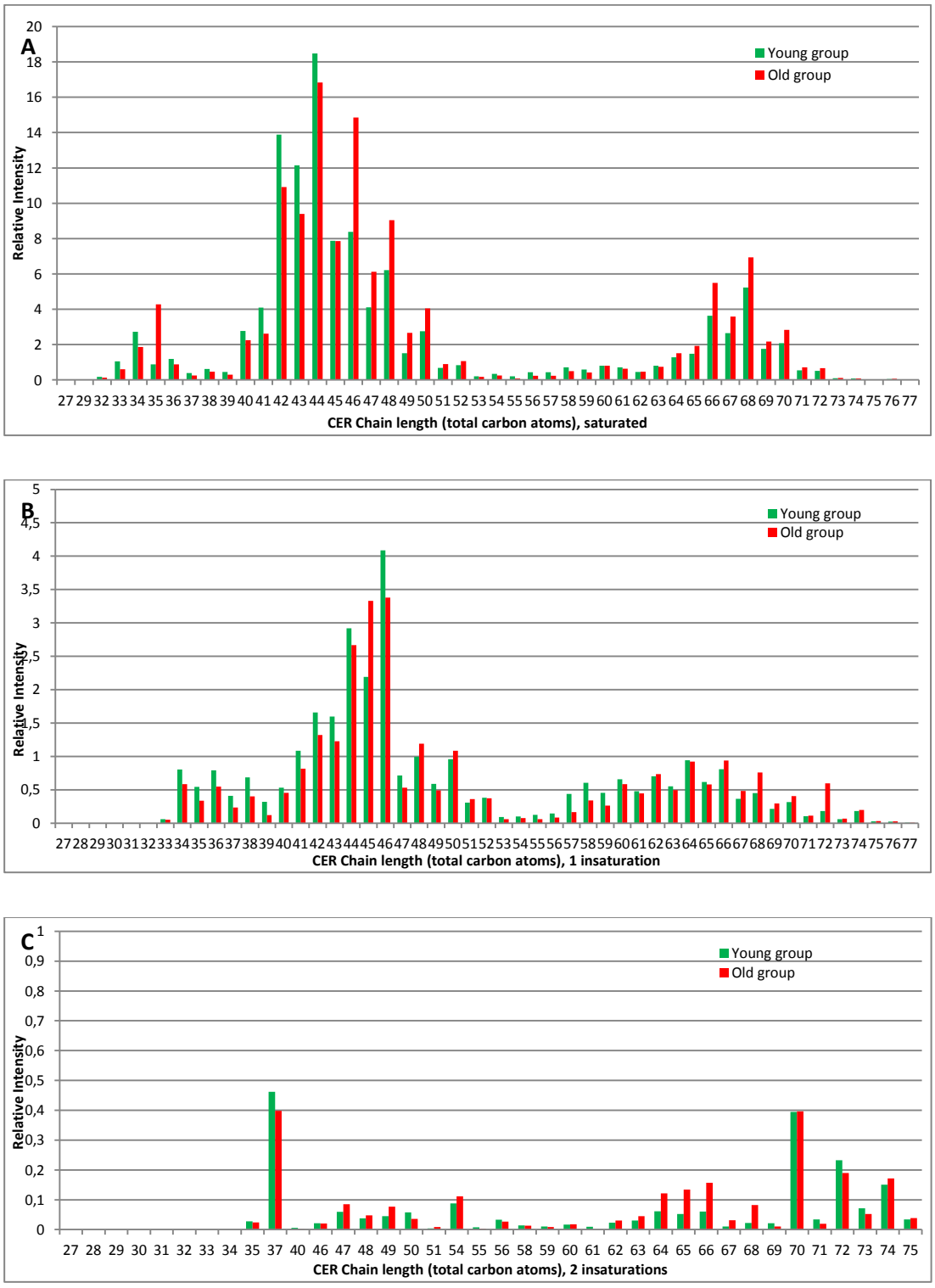


Fig. 5. *The distribution of the relative abundances of the CER chain length for the a) saturated, b) monounsaturated, c) diunsaturated ceramides. The CER chain length distribution shows that independent of the age groups the VLC carbons are dominant in each distribution and the ULC carbons are extremely long. In the case of the diunsaturated the distribution is not well-defined.*

***TABLE 4**. The relative contribution of each carbon chain length ceramide ranges in the saturated, monounsaturated and diunsaturated ceramides subclasses are given.*

Young group	Contribution of LC, (%)	Contribution of VLC, (%)	Contribution of ULC, (%)
0 ins	5.8	75.9	18.3
1ins	9.9	55.4	34.6
2ins	23.6	18.5	57.9

Old group	Contribution of LC, (%)	Contribution of VLC, (%)	Contribution of ULC, (%)
0 ins	6.5	71.8	21.8
1ins	7.4	59.6	33.0
2ins	18.0	18.7	63.2

***TABLE 5.** The subclasses ceramides with major signal and the most numerous subclasses ceramides depend on the carbon chain length. The subclasses with major signal and the most numerous subclasses are considered for the saturated, monounsaturated and diunsaturated ceramides subclasses, similar for young and old groups.*

Saturated ceramide	Subclasses with major signal	Most numerous subclasses
LC	NH	AH
VLC	NH	AP
ULC	EOH	(1-O-E)AdS

monounsaturated ceramide	Subclasses with major signal	Most numerous subclasses
LC	AT	AT
VLC	AP	AP
ULC	(1-O-E)AdS	(1-O-E)AdS

diunsaturated ceramide	Subclasses with major signal	Most numerous subclasses
LC	(1-O-E)NdS	(1-O-E)AdS and (1-O-E)NdS
VLC	(1-O-E)NS	(1-O-E)NS
ULC	EOP	(1-O-E)AdS and EOP

Molecular species that are over-expressed in the young or in the old groups

Semi quantitative approach was applied: firstly, relative ratio $I_{species}$/ $I_{internal\ standard}$ and secondly and ratio between mean $I_{18\text{-}30}$/ $I_{65\text{-}75}$ were calculated for each molecular species. The molecular species showing the most differences have been selected with a mean ratio $I_{18\text{-}30}$/ $I_{65\text{-}75}$ higher than 5, in the same way for the mean ratio $I_{65\text{-}78}$/ $I_{18\text{-}30}$. All are shown in **Fig. 6** with their average intensity. The inter age groups differences represent about 40 molecular species: near 75% higher in the young group and near 25% higher in old group. The highest intergroup differences are observed in the dS-CER and the T-CER classes. The main of them belong to dS-CER subclass, more precisely 1-O-acylceramides (3 alkyl chains) with α-hydroxylated fatty acids chain, (1-O-E)A. The alkyl chain is quite longer for the old group. H-CER class presents no intergroup difference. For the same ceramides class, the same insaturation numbers, the most abundant species have longer chains in the old group compared to the young group.

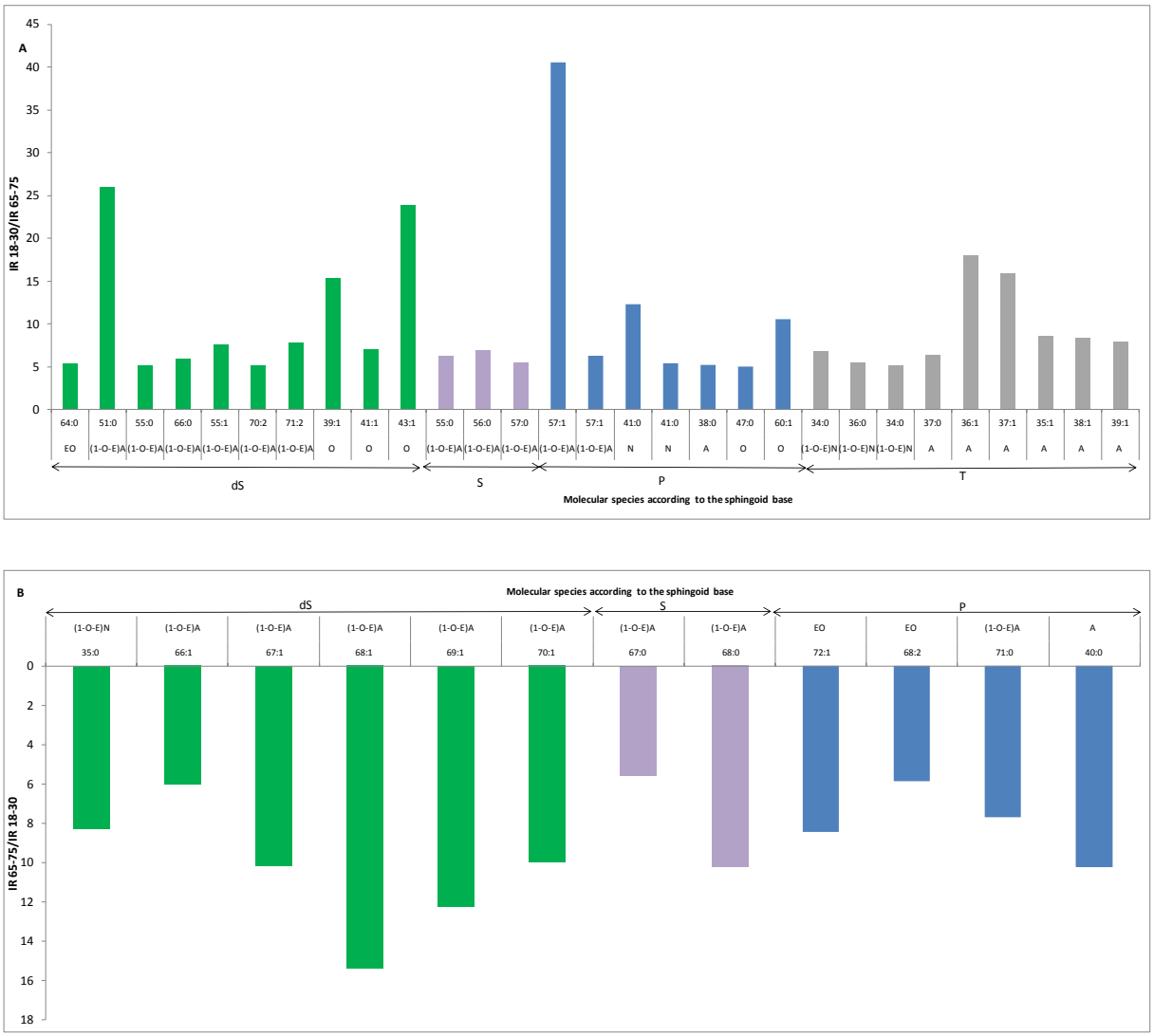


Fig.6. The most representative molecular species that are at the origin of the discrimination between younger and older groups. a) molecular species for the younger group, b) molecular species for the older group. dS-CER is shown in green, S-CER in purple, P-CER in blue, and T-CER in grey.

DISCUSSION

The goal of this study was to assess the age- and site-dependent changes of the lipid composition and lateral organization at the uppermost layer of the SC. To that end an *in vivo* investigation was conducted on 20 European Caucasian women divided into 2 age groups: 18-30 and 65-75 years of age. Three skin sites of interest were compared: face (cheek), exposed arm site (dorsal forearm), and protected arm site (upper inner arm). The two arm sites were selected to explore potential differences between chronological and photoaging aging effects on the lipid composition and organization. is the face site was selected as physiologically and structurally different from the arm skin. Our data show that lipid composition and lipid organization is only slightly modulated with the age of the person but strongly depends on the skin site location.

Does the lipid content evolve with age?

We reported that the lipid content weakly decreases with age mainly on the face and the exposed arm site. Thus the change in lipid level seems to be more related to photoaging. This limited decrease of the total lipid content is compensated by an increase of the SC thickness with age (17). Effectiveness of the SC water barrier function depends on its intercellular lipid content and composition, but also on the size of corneocytes. As the desquamation rate decreases with age the time the corneocytes spend in the SC increases leading to an increase of the corneocyte size. In terms of barrier efficacy the increase in size may compensate for the lipid depletion with aging. The preservation of the SC barrier function may also be in part due to the SC maintaining the relative proportions of the lipids present(43).

How does the lipid organization evolve with age?

In terms of lipid lateral organization, packing fluidity was found to decrease with aging,

as the spectral position of the peak of the CH_2 symmetric stretching bond near 2848 cm^{-1} shifted towards lower frequency with age. Analysis of the IR spectra showed that lipid organization at the upper-most part of the SC appears to become more orthorhombic with aging. Prevalence of the more tightly packed orthorhombic phase has been shown to correlate with higher SC integrity and barrier efficiency as measured by TEWL ((44)).

Does the lipid organization depend on the skin site?

Lateral lipid organization on the upper-most layers of the SC on the face appears to be less compact than on the two arm sites. The wavelength shift of the peak of the CH_2 symmetric stretching band near 2849 cm^{-1} was longer on the face than on the arm sites and this observation was independent of the age group. Moreover, the SC is thinner on the face than on the two arm sites and taken together with the SC lipid organization, it explains the observed weaker barrier function on the face (17). The ATR-FTIR method collects lipid signals that originate both from within the SC and from sebum on the skin surface. The signal at 1740 cm^{-1} is characteristic of triglycerides that are part of the sebaceous lipids. The ratio $\nu_{C=0\ \text{stretching band of lipid ester carbonyl}} / \nu_{CH\ \text{stretching vibration band of protein}}$ is higher on the face than on the two arm sites indicating a higher sebum content on the face as expected due to the higher activity of the sebaceous glands on the face.

Why do we use this method to identify ceramide classes and ceramide subclasses?

Extracted lipids in a non-invasive way by using cotton swab have been used instead of tape stripping because it has been previously reported less interferences due to polymers and good recovery.

Over the years, few methods have been developed from High Performance Thin Layer Chromatography (HPTLC) tandem to Mass Spectrometry to Liquid Chromatography combined to Mass Spectrometry (45–54). In our experiment, the Normal Phase Liquid

Chromatography (NP-LC) combined with an Atmospheric Pressure Chemical Ionization Source (APCI) offers some advantages compare to the Reversed-Phase Liquid Chromatography (RP-LC) combined with an Electrospray Ionisation (ESI): the lower ion suppression (55, 56) and the possibility to separate the different classes of ceramides due to their polarities of the head group. Moreover, the higher flow rates are compatible with an APCI source compared with an ESI source resulting in a shorter time analysis. As the variations in the structure of ceramides are huge, it is important to use a normal phase separation of the ceramides to assign the correct structures of the compounds in one class. Moreover in our study we used a PVA-sil polar stationary phase that gives advantage in terms of separation and peak shape compared with commonly used silica columns (57, 58). Internal normalization has been used to compare ceramides classes and subclasses. An internal standard is only used to compare the ceramides entities content between the old and young volunteers. This method enables to semi-quantify the abundances of the individual ceramides.

The distribution of the ceramides depends both on sphingoid base and fatty acid moiety

The total ceramide content decreases with aging. We found that the sum of the H-CER represents about 30% in the human SC. T'Kindt et al. and Van Smeden et al. (59) also found a similar result to us (26). Stewart and Downing also showed a significant amount of H present in human skin (60). The origin of the H-CER is not yet fully understood. It seems that a hydroxylase similar might hydroxylate S-CER as the allylic position C-6 is prone to oxidation. The H-CER are found in the human epidermis (61) and not in all mammalian epidermis leading to some difficulties to know the exact role of the H-CER in skin barrier function. However, some studies reported consistently lower amount of H-CER in atopic skin compared to the normal skin (62). This leads to the importance of the H-CER in the skin barrier function. The additional hydroxyl group could possibly serve to strengthen the number of H-bonds leading to a better resistance to external stressors and increased cohesion of the SC lipid lamellae.

The action of the dihydroceramide desaturase 2 on the dihydroceramide to produce the P-CER could be slightly modified with aging as the P-CER content decreases with aging. The ceramides are produced in two different ways: *a de novo* pathway and a salvage pathway (23, 63–65). The *de novo* pathway lets the production of the dihydrosphingosine leading to dS-CER. Then by the action of the dihydroceramide desaturase 1 and 2, the S-CER and P-CER are respectively produced. The salvage pathway leads to the production of the S-CER, P-CER, T-CER and the H-CER, the ceramides that are directly produced from sphinganine (66).

Since the portion of S-CER does not differ between the two age groups, it can be assumed that the action of the dihydroceramide desaturase 1 on the dihydroceramide is not modified with aging. Moreover, since the percentage of S-CER does not depend on the age group, it can be linked to the conservation of the skin barrier function with aging. Mizutani et al. (64) also reported that the S-CER are essential in the skin barrier function. Moreover, Rerek et al. (67) show that the S-ceramides have an orthorhombic organization letting a very dense imbrication within the lipid matrix contrary to the P-ceramides, responsible for a hexagonal organization.

The three most abundant ceramides subclasses are CER(NP), CER(NH), and CER(AP)(59). Their sum is similar for the two groups (about 50%). An earlier study in 1991 by Yamamoto et al. (68) showed that the three most important subclasses were CER(AS) with 26.3%, CER(AP) with 21.9% and CER(NP) with 20.5%. However later studies by Masukawa et al. (53) in 2009, Ishikawa et al. (69) in 2010, Janssens et al. (9) in 2011 and t'Kindt et al. (26) in 2012 are in agreement with our finding. The discrepancy with the earlier study can be explained by the few numbers of known subclasses were investigated and this could explain why the percentages were increased.

Only the percentage of CER(NP) decreases with age. Decreases in CER(NP) content have been found to contribute to an abnormal permeability barrier (70–72). The percentage of CER(NH) increases with aging. The **Fig.3** shows that the ratio

CER(NH)/CER(NP) increases with age and in a general way, the ratio H-CER/ P-CER increases with age. A hypothesis could be that the increase of H-CER with aging supports the increased cohesion of the SC lipid lamellae and could compensate for the decrease of the SC total lipid content.

Globally, the percentage of the ceramide with an esterified fatty acid moiety increases with age. It was found that the formation of the LPP is always observed with the presence of acylceramides. The presence of the CER(EOS) in the structures is necessary (38, 73) to create space that can be filled by long chain fatty acids (37) making the structure more tightly packed. Although its relative abundance is low, CER(EOS) is important as it promotes the formation of the LPP more efficiently than CER(EOP)(38). The resulting structure is more compact, with decreased packing fluidity (37). Thus it contributes to a better barrier function with age. At the same time, the CER(EOH) content increases with aging making the polar head group region of the molecule larger compared to EOS. This will require greater movement of the aliphatic chains under the polar head group, which would make the membrane domain more fluid and, therefore more permeable. Whereas highly ordered membrane domains will be favorable for impermeability, some degree of fluidity is also necessary for flexibility of the lipid lamellae. This demonstrates the necessity to have the CER(EOS), CER(EOP) and CER(EOS) to balance the requirement for a relatively impermeable skin with the need for flexibility.

The skin aging does not affect the number of unsaturation on the total carbon chain length

The most abundant ceramides in the SC of the older group have 44/46 total carbon atomswhile for the younger group the most abundant species have 42/44 total carbon atoms. Interestingly, the ceramide chain length with an even number of carbon is more abundant than those with an odd carbon number. These results are coherent with the

findings of Van Smeden et al. (59)

The results concerning the trimodal distribution observed for the saturated and monounsaturated are in accordance withWertz and Nòrlen (2003) who found that the most characteristic of the ceramides are 1) the compositional heterogeneity with a broad chain length distribution in the FA moiety of CER (C20 to C32, peaking at C24) corresponding to C40 to C52 total carbon atoms in the ceramide chain for a C20 sphingoid base and 2) the prevalence of saturated VLC hydrocarbons (C20:0-C32:0) corresponding to C40 to C52 total carbon atoms in the ceramide chain.

Concerning the diunsaturated ceramides (ω-esterified ceramides containing a linoleic acid moiety and mainly CER(EOP)), not all chain lengths are represented and the trimodal distribution is not well defined compared to the saturated and monounsaturated ceramides.

CONCLUSION

Aging affects only slightly the total lipid content and organization at the uppermost layers of the SC. SC of the older group contains fewer lipids and less ceramides. Independent of age the SC lipids are globally organized in an orthorhombic phase. Only a few variations were observed with age, linked to the slight modifications of the micro-heterogeneity of the ceramides in the lipid matrix. The variations only represent 2% of molecular species. The SC lipid compactness is slightly higher with age probably due to an increase in the carbon chain length and the relative amount of H-CER/P-CER. This increase in lipid compactness leads to a decrease in packing fluidity with age. Taken together these results help explain how the skin barrier function is maintained with aging in healthy skin.

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CHAPITRE:

3 HYDRATATION CUTANÉE ET VIEILLISSEMENT

I HYDRATATION CUTANÉE

Changements avec l'âge dans le SC : Composition NMF et teneur en eau

Mobilité des molécules d'eau dans le SC

1 | Contexte

L'eau a une contribution secondaire sur la qualité de l'organisation des édifices lipidiques, mais en réalité, sa présence au sein du SC est très importante car elle joue un rôle majeur dans le maintien de l'homéostasie cutanée (Rawlings and Matts 2005). Il contient des molécules appelés facteurs naturels d'hydratation, NMF comprenant principalement l'alanine, la glycine, l'histidine, l'arginine + l'ornine + la citrine, la proline, la sérine, le lactate, l'urée, l'acide trans-Urocanique noté tUCA, et l'Acide Carboxylique Pyrrolidone noté ACP (Clar and Fourtanier 1981; Rawlings and Harding 2004) ainsi que des sucres et des ions minéraux. Les NMF sont des substances polaires, hydrosolubles et hygroscopiques contenues dans les cornéocytes (Marty 2002) et formés lors de la différenciation des kératinocytes par hydrolyse des protéines comme la filaggrine. Ces NMF sont à l'origine de la rétention d'eau du fait de leur affinité pour les molécules d'eau dans le SC. Ils se trouvent avec les molécules d'eau dans les parties protéiques du SC c'est-à-dire au niveau des amas de kératine. Les NMF maintiennent le degré d'hydratation cutanée (Rawlings and Matts 2005) à un niveau optimal aidant ainsi à la fonction barrière hydrique (Verdier-Sévrain and Bonté 2007) et à la qualité élastique du SC (Blank 1952). Même si le SC contient peu d'eau, celle-ci joue un rôle primordial dans la physiologie cutanée car elle contrôle des réactions enzymatiques responsables de la cornédesmolyse et de la desquamation. L'eau joue également un rôle cosmétique. Lors du vieillissement, la teneur en eau totale dans le SC peut être normale (Thune 1988; Cua et al. 1990; Eisner et al. 1990; Wilhelm et al. 1991) ou bien être diminuée

légèrement (Potts et al. 1984; Jackson et al. 1993; Mc Callion and Po 1993; Harvell and Maibach 1994) parce que la capacité de rétention d'eau du SC diminue notamment à cause de la baisse de la production des lipides (Mc Callion and Po 1993);(Imokawa et al. 1986).

En micro-spectroscopie confocale Raman peut être calculée par l'intermédiaire d'algorithmes mathématiques, la teneur en un bon nombre de NMF. L'eau totale contenue dans la couche cornée est mesurée classiquement par cornéométrie, et plus récemment par micro-spectroscopie confocale Raman. La cornéométrie donne un critère global limité à la surface de la couche cornée dont la mesure dépend beaucoup de la concentration ionique en surface. A contrario, la micro-spectroscopie confocale Raman est beaucoup plus informative. Non seulement, elle donne une information pertinente en profondeur mais également elle permet de quantifier les acteurs majeurs de l'hydratation du SC c'est-à-dire eau et NMF de façon distincte.

Au plan fondamental, de nombreuses équipes (Bulgin and Vinson 1967; Takenouchi et al. 1986; Gniadecka et al. 1998; Kasting et al. 2003; Pieper et al. 2003; Yadav et al. 2007; Nakagawa et al. 2011) ont travaillé sur la différenciation des mobilités des molécules d'eau. Takenouchi et al. (Takenouchi et al. 1986) ont défini trois types d'eau : l'eau fortement liée, l'eau partiellement liée et l'eau non-liée. Aucune méthode jusqu'à présent utilisée pour déterminer la teneur en eau dans la couche cornée, comme la Calorimétrie Différentielle (DSC) ou encore la micro-spectroscopie confocale Raman, n'avait permis de distinguer l'eau liée, l'eau partiellement liée et l'eau non-liée. Ces différentes informations ont été utilisées pour la compréhension de la mobilité de l'eau dans le SC. En 2013, Vyumvuhore et al. (Vyumvuhore et al. 2013) ont utilisé la micro-spectroscopie Raman pour définir *ex vivo* les bandes caractéristiques de ces trois états de l'eau. L'eau peut être auto-associée, reliée à des structures protéiques ou reliée à des structures des édifices lipidiques (**Figure 11**). Selon la quantité relative de ces trois catégories de mobilité d'eau dans la couche cornée, le SC aura des caractéristiques physiques différentes.

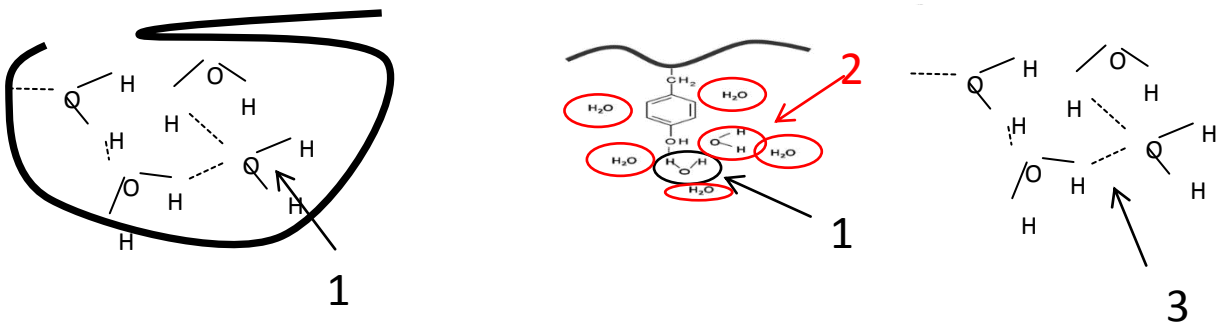

Figure 11 : Structure des molécules d'eau. *Selon leur mobilité les molécules d'eau dans le SC sont réparties selon trois catégories :*

L'eau fortement liée ou liée ou constitutive « 1 »*: les molécules d'eau forment des liaisons hydrogènes fortes avec les protéines et les autres molécules du SC. Ces molécules sont les moins mobiles. On pourrait caractériser ces molécules comme représentant la teneur minimale en eau dans la couche cornée.*

L'eau partiellement ou secondairement liée « 2 » *: les molécules d'eau forment des liaisons hydrogènes avec les molécules d'eau liée et peuvent être des liaisonssecondaires ou tertiaires ou d'un ordre plus grand. Elles forment un « nuage » autour du site de liaision et sont d'une mobilité intermédiaire. L'eau partiellement liée aura un impact sur l'organisation des édifices lipidiques du SC.*

L'eau non-liée ou mobile ou autoassociée « 3 » *: les molécules d'eau forment des liaisons labiles avec les autres molécules d'eau et peuvent diffuser à travers le SC. Elles sont toujours dans un état liquide et peuvent migrer assez rapidement vers la surface de la couche cornée.*

Les effets du vieillissement cutané et du photo-vieillissement sur les propriétés cutanées et notamment l'hydratation ne sont pas complètement éclaircis et la littérature rapporte des résultats contradictoires. Il est donc nécessaire de faire l'investigation des effets du vieillissement chronologique et du photo-vieillissement au niveau moléculaire à travers la teneur en eau et la composition des NMF dans la couche cornée. De même, la quantité relative en fonction de la profondeur des trois catégories de mobilité d'eau dans le SC des personnes jeunes et âgées n'a jamais été explicitée. Ainsi, notre étude a porté sur les effets du vieillissement cutané sur l'état d'hydratation cutanée : teneur en eau globale et NMF et la mobilité de l'eau dans la couche cornée en se focalisant sur les effets de l'âge et de l'exposition chronique à l'environnement.

2 | Méthode

Dans ce travail, des mesures *in vivo* sur un ensemble de femmes caucasiennes âgées de 18 à 70 ans ont été réalisées à Température et Humidité Relative contrôlées (20-25°C, 40% RH) sur trois sites corporels pour étudier l'hydratation cutanée selon le site corporel dans les tissus cutanés superficiels, le SC: 1) face, partie corporelle qui a un comportement physiologique cutané différent et qui est l'objet de soins cosmétiques particuliers et plus fréquents que ceux effectués sur le reste du corps, 2) bras exposé au soleil, appelé également avant-bras face postérieure (photo-vieillissement) et 3) bras protégé du soleil, appelé bras face antérieure (vieillissement chronologique). Les mesures ont principalement été réalisées par micro-spectroscopie confocale Raman afin d'obtenir une information aux niveaux moléculaire et supramoléculaire.

Au niveau tissulaire, l'épaisseur de la couche cornée a été déterminée comme précédemment à partir du profil de concentration de l'eau, (Egawa et al. 2007; Egawa and Tagami 2008; Bielfeldt et al. 2009) ceci afin de normaliser les profils de concentration moléculaires par rapport à l'épaisseur du SC pour l'ensemble des volontaires.

Au niveau moléculaire, la teneur en chacun des composés NMF pour chaque profondeur (0 à 24 µm avec un pas de 4 µm) et en eau pour chaque profondeur (0 à 32 µm avec un pas de 4 µm) ont été établies. La teneur en chacun des NMF a été calculé à partir d'algorithmes mathématiques développés par River Diagnostic (Caspers et al. 2001). Les concentrations en NMF (comprenant principalement alanine, glycine, histidine, arginine + ornine + citrine, proline, sérine, lactate, urée, l'acide trans-Urocanique, et l'Acide Carboxylique Pyrrolidone) ont été mesurées dans la région spectrale comprise entre 400-1800 cm^{-1} (Caspers et al. 2001). La teneur en eau est obtenue par le critère de River Diagnostics $\nu_{O\text{-}H,\ (3350\text{-}3550\ cm\text{-}1)}/\nu_{CH2,\ (2910\text{-}2965\ cm\text{-}1)}$ (**Figure 12**). Les profils de concentration des NMF et de l'eau ont ensuite été établis en fonction de la profondeur. La teneur totale en chacun des composés est ensuite obtenue par intégration des profils de concentration normalisés depuis la surface du SC jusqu'à la jonction SC-SG.

Au niveau supramoléculaire, la mobilité des molécules d'eau est impliquée dans la compacité des édifices lipidiques par l'intermédiaire d'un nombre plus ou moins élevé de liaisons H. Vyumvuhore et al. (Vyumvuhore et al. 2013)ont étudié la région spectrale comprise entre 3100 et 3700 cm^{-1} de façon approfondie. La bande de l'eau a été déconvoluée en quatre sous-bandes afin de déterminer les vibrations spécifiques des différents états de l'eau. Il en a résulté trois zones spectrales (**Figure 13**) sur les spectres Raman correspondant à l'eau fortement liée (3150-3245 cm^{-1}), l'eau partiellement liée (3245-3420 cm^{-1}) et l'eau non-liée (3420-3600 cm^{-1}). Les trois mobilités de l'eau ont donc été étudiées en utilisant ces trois bandes en micro-spectroscopie confocale Raman.

En addition, des mesures biométriques ont été réalisées, en particulier la conductance cutanée, qui caractérise classiquement et de façon globale l'hydratation de la surface de la peau.

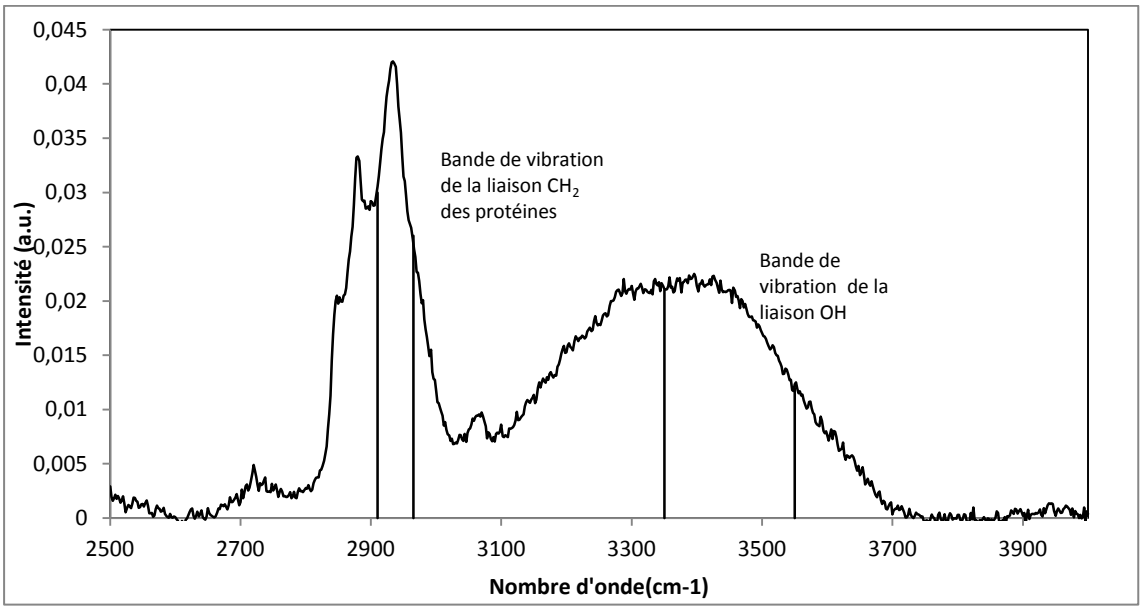

Figure 12 : Bande spectrale de vibration de la liaison CH_2 des protéines et de la liaison OH dans la région 2500- 4000 cm^{-1} *avec la bande caractéristique de la liaison CH_2 (2910-2965 cm^{-1}) des protéines et la bande caractéristique de la liaison OH (3350- 3550 cm^{-1}), critère donné par River Diagnostics pour le calcul de l'eau (Caspers et al. 2000).*

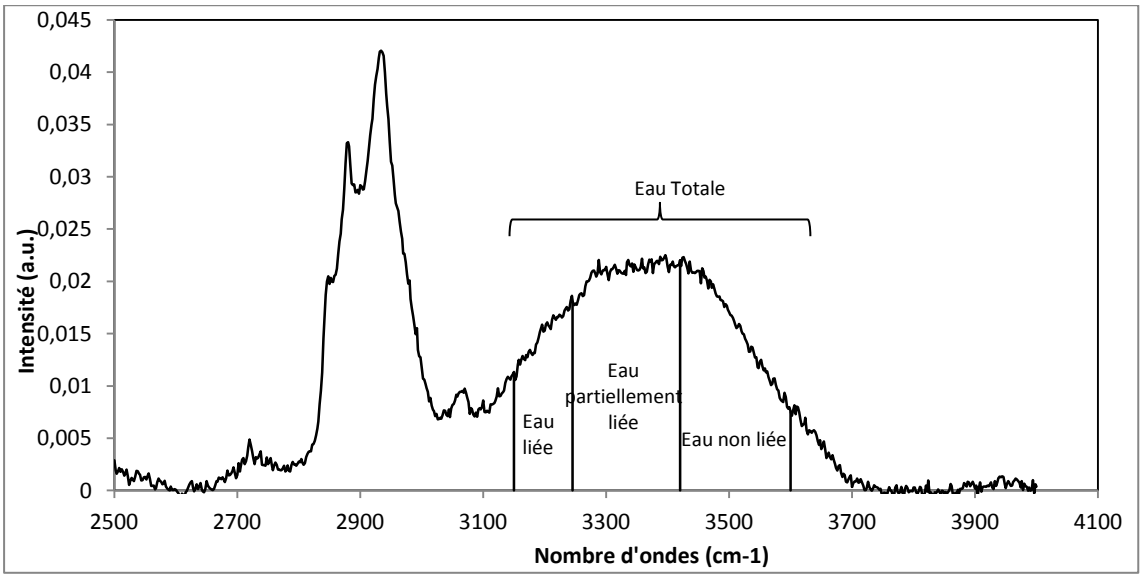

Figure 13 : Bande spectrale des différents états de mobilité de l'eau : *eau liée (3150-3245 cm^{-1}), eau partiellement liée (3245-3420 cm^{-1}) et eau non-liée (3420-3600 cm^{-1}) selon Vyumvuhore et al. (Vyumvuhore et al. 2013).*

3 | Résultats

Les résultats de conductance ont montré que le taux d'hydratation à la surface de la couche cornée est constant avec l'âge. Cette méthode est peu sensible aux variations de l'hydratation mais ce constat traduit certainement de très faibles modifications si elles existent.

La micro-spectroscopie confocale Raman plus pertinente a au contraire donné l'information d'une variation de la teneur en NMF et en eau avec l'âge selon les résultats résumés dans le tableau (**Tableau 3**). Le profil de concentration moyen des NMF montre un plateau de l'épiderme profond jusqu'à la jonction SC-SG, puis une augmentation de la jonction vers la surface cutanée. Cette dernière reflète les différents processus protéolytiques le long de la couche cornée de l'épiderme vers la surface. Lors de la migration des cornéocytes vers la surface de la peau, les protéines de la famille S100 (Filaggrine, Filaggrine-2 et hornerine) sont protéolysées en NMF (Harding et al. 2003);(Scott et al. 1982). Parallèlement, le profil de l'eau mesurée, quelque soit l'âge montre tout d'abord un plateau de l'épiderme profond s'arrêtant à la jonction SC - SG puis un gradient inverse à celui du profil de concentration des NMF vers la surface cutanée. L'analyse de ces profils (NMF et eau en fonction de la profondeur) montrent donc qu'ils sont inversement corrélés, ce qui laisserait supposer que les NMF ont un rôle compensateur vis-à-vis de la diminution de l'eau vers la surface de la couche cornée.

Il y a une augmentation générale des NMF et en particulier des amino-acides avec l'âge sur tous les sites corporels mais statistiquement significative sur la joue. Elle peut être dûe à un ralentissement du renouvellement cellulaire avec l'âge et un épaississement de la couche cornée en vieillissant. La teneur en eau quant à elle, diminue légèrement avec l'âge et de façon significative sur le site exposé du bras. Le vieillissement cutané a donc peu d'impact sur l'état d'hydratation cutanée. Cependant, l'exposition solaire au cours de la vie influence en final cet état.

Nous avons vu précédemment que la force des liaisons H entre molécules d'eau et têtes

polaires des lipides joue un rôle dans la compacité des édifices lipidiques. Il est donc très intéressant d'étudier la variation des trois états de mobilité de l'eau dans la couche cornée. Pour l'ensemble des volontaires, les pourcentages des trois états de l'eau par rapport à la teneur en eau totale (3150 cm^{-1}– 3600cm^{-1}) quelque soit la profondeur de mesure (SC ou SG) sont de l'ordre de (**Figure 14**):

- 17% d'eau liée constitutive du tissu cutané
- 34 % d'eau non-liée auto-associée
- 49 % d'eau partiellement liée, liée aux protéines et aux lipides

La teneur en eau non-liée et en eau fortement liée diminuent vers la surface. Il y a également une augmentation de la teneur en eau partiellement liée. En effet, la somme des pourcentages doit être égale à 100%. Ces variations peuvent être reliées à la variation en surface des NMF.

Les modifications observées sont extrèmement subtiles et plusieurs hypothèses peuvent être émises. Tout d'abord, la desquamation intervient de façon majeure ce qui entraine une diminution de l'eau liée au niveau des couches les plus externes des cornéocytes. De plus, l'eau auto-associée s'évapore plus facilement vers la surface. La somme des pourcentages des trois états de mobilité de l'eau étant égale à 100%, le pourcentage d'eau partiellement liée augmente. En parallèle, on a une augmentation des NMF vers la surface cutanée qui est corrélée avec cette augmentation d'eau partiellement liée. Une autre explication serait que l'eau liée est liée à la tête polaire des lipides intercornéocytaires (Yadav et al. 2007). La teneur des lipides diminuant dans les couches très supérieures du SC, cela conduirait à une diminution de l'eau liée.

De plus, il est intéressant de constater qu'un profil de concentration similaire à celui de l'eau totale est observé pour chacun des trois états de mobilité de l'eau : un plateau de l'épiderme profond jusqu'à la jonction entre le SC-SG, puis un gradient de concentration qui diminue de la jonction vers la surface cutanée.

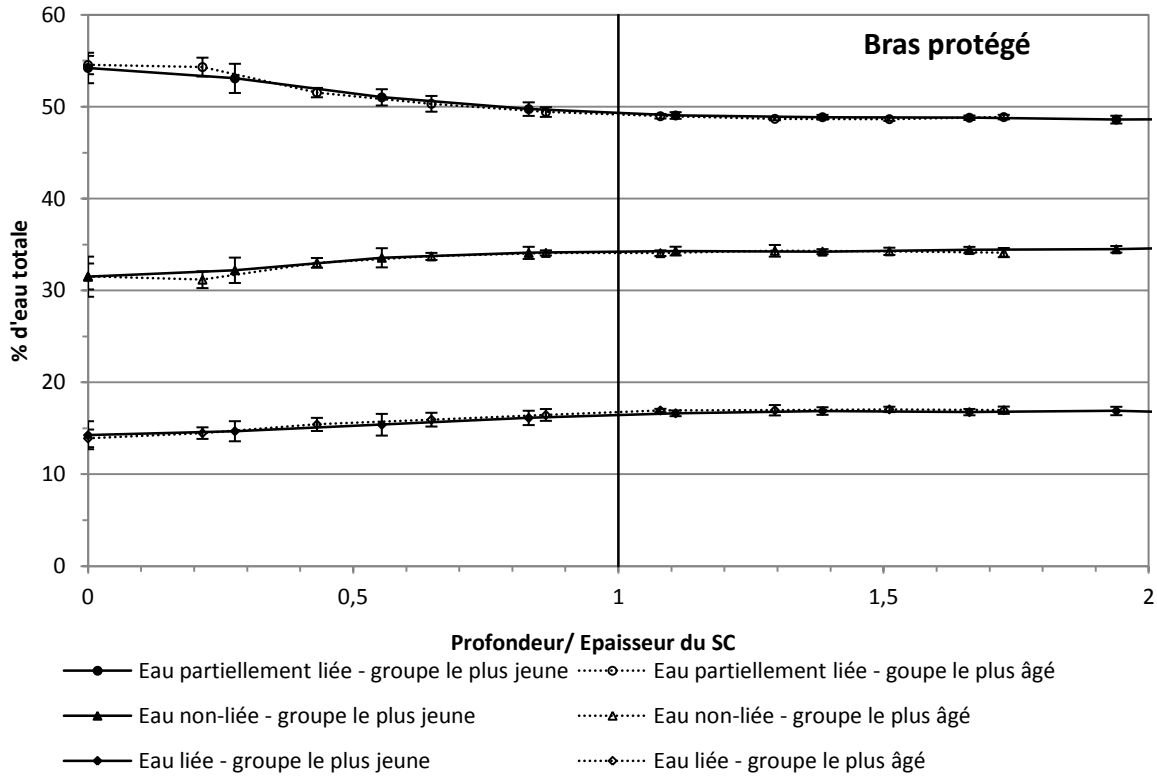

Figure 14 : Distribution des trois états de mobilité de l'eau pour le groupe des personnes jeunes et âgées sur le bras protégé. *Cette distribution des trois états de l'eau selon leur mobilité est indépendante de l'âge et du site corporel, les données ici représentent le site du bras protégé.*

L'hydratation à la surface cutanée du SC ne montre pas de différence entre la joue et les deux autres sites corporels. Quelque soit l'âge, les concentrations globales en NMF ne montrent pas de différence entre la joue et les deux autres sites corporels bien qu'il apparaisse des différences d'évolution de concentration selon les molécules NMF considérées. D'autres sources de protéines du SC autre que la filaggrine, comme l'hornerine et la capsase-14 sont sans doute impliquées dans la production de NMF lors du processus de cornification (Scott et al. 1982; Rawlings and Harding 2004; Kamata et al. 2009). Elles peuvent être hydrolysées (Scott and Harding 1986; Rawlings et al. 1994) et agir comme une source de certains NMF ce qui expliquerait les différences d'évolution moléculaire.

De façon intéressante, quelque soit l'âge, la teneur en lactate apparait plus importante sur la joue comparée aux bras. Le lactate est connu pour avoir un effet hydratant (Nakagawa et al. 2004). Le lactate résulte du processus métabolique anaérobique des cellules de la couche basale de l'épiderme et peut diffuser à travers le SC (Heiden et al. 2009). Le taux de renouvellement cellulaire élevé du SC de la joue peut expliquer le contenu supérieur de lactate en comparaison avec le SC des deux autres sites corporels. La teneur en eau dans le SC de la joue est plus importante que dans le SC des deux autres sites corporels du bras. Le SC de la joue est plus fin mais le taux de renouvellement cellulaire est plus grand (Stamatas et al. 2006; Boireau-Adamezyk et al. 2014). Les habitudes cosmétiques peuvent également expliquer la différence entre les sites corporels.

En ce qui concerne la mobilité de l'eau, les mêmes profils de concentration sont observés sur les trois sites corporels et sont toujours similaires à celui de l'eau totale: un plateau de l'épiderme profond jusqu'à la jonction entre le SC-SG, puis le gradient de concentration diminue vers la surface cutanée. Les profils de concentration des trois catégories de mobilité de l'eau exprimé en % par rapport à la teneur en eau totale conduit à des profils de concentration similaires quelque soit les sites corporels (**Figure 14)**: eau fortement liée < eau non-liée < eau partiellement liée.

Tableau 3 : Tableau récapitulatif des paramètres comprenant les différents NMF, et l'eaumesurés par micro-spectroscopie confocale Raman *et leur dépendance selon l'âge et le site corporel.*

Paramètres	Age	Site corporel (joue vs bras)	Exposition au soleil (bras exposé vs bras protégé)
[Eau Totale]	Diminue sur le site du bras exposé	Supérieure sur la joue	Pas de différence
[NMF Total]	Augmente sur la joue	Pas de différence	Pas de différence
[AA Total]	Augmente sur la joue	Pas de différence	Pas de différence
[Lactate]	Diminue sur le site du bras protégé et sur le site du bras exposé	Supérieure sur la joue	Pas de différence
[Ala]	Augmente sur la joue et sur le site du bras exposé	Pas de différence	Pas de différence
[Gly]	Augmente sur le site du bras protégé	Inférieure sur la joue	Pas de différence
[His]	_	Pas de différence	Pas de différence
[Orn]	Diminue sur le site du bras protégé	Pas de différence	Pas de différence
[Pro]	Augmente sur la joue et diminue sur le site du bras protégé	Pas de différence, excepté pour les deux derniers groupes d'âge, supérieure sur la joue	Pas de différence
[Ser]	Augmente sur le site du bras protégé et sur le site du bras exposé	Inférieure sur la joue	Pas de différence
[Urée]	Diminue sur le site du bras protégé	Pas de différence	Pas de différence
[tUca]	_	Pas de différence excepté sur la joue pour le groupe le plus jeune: inférieure sur la joue	Pas de différence
[ACP]	_	Pas de différence excepté sur la joue pour le groupe le plus jeune: inférieure sur la joue	Pas de différence

4 | Conclusion

L'état d'hydration du SC est caractérisé par la teneur en eau totale, la teneur et la composition en NMF et les proportions relatives de chaque état de mobilité de l'eau. Les différents états de mobilité de l'eau semblent très stables avec le temps et quel que soit le site corporel. Le photo-vieillissement n'amplifie pas les phénomènes. Nous n'avons pas observé de variations majeures de ces paramètres au cours du vieillissement cutané des peaux saines. Les changements mineurs observés peuvent être expliqués par des variations de processus enzymatiques à l'origine de la desquamation ou de la production de NMF. Ainsi, la fonction barrière hydrique indispensable au processus vital est maintenue lors du vieillissement.

II ARTICLE 3

Age-dependent changes in stratum corneum - Part II: water content and natural moisturization factors

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ABSTRACT

Background/purpose The objective of this study was to examine if age and chronic environmental exposure affect the water content and the composition of the Natural Moisturizing Factor (NMF) of the Stratum Corneum (SC).

Methods The study was conducted on 40 French women volunteers without history of skin diseases. The volunteers were selected to cover the age range 20-70 years. Measurements were done on the cheek and on two skin sites on the arm (one relatively protected and one exposed). SC water content and NMF composition (alanine, glycine, histidine, arginin+ornine+citrine, proline, serine, lactate, urea, trans urocanic acid, pyrrolidone carboxylic acid, total NMF, and total Amino acid) was measured by Raman confocal microspectroscopy. Skin surface hydration was measured by skin conductance.

Results The SC water content was slightly reduced with age, a change that reached statistical significant levels only on the exposed arm site. Moreover, the SC on the face contains significantly more water than the SC on the two arm sites tested. The individual amino acid concentrations showed species-dependent behaviors with age: some increased, some decreased, and some remained constant, potentially indicating different protein sources. Interestingly, on the arm sites the sum of decreasing amino acid concentrations is compensated by the sum of those increasing, resulting in constant total amino acid content. However on the face the total amino acid content statistically increased with age potentially relating to the declining cell turnover rates. The lactate content was higher on the face for all ages and statistically decreased on both arm sites.

Conclusion Both chronological aging and chronic exposure to environmental factors mildly affect SC hydration, while they have variable effects in the concentrations of NMF components. The dynamics of NMF composition may at least partially explain the age-related changes in SC hydration.

INTRODUCTION

The Stratum Corneum (SC) is the outermost epidermal layer which protects the human body against environmental insults, such as pro-oxidants, microorganism invasion, and dehydration to name a few. The SC consists of proteins, lipids, and water. The proteins are highly cross-linked and are mostly located inside the corneocytes and on corneodesmosomes. The corneocytes are surrounded by a lipid matrix consisting of free fatty acids, cholesterol, and ceramides (1). This “brick and mortar” model explains the efficient barrier function that protects the human body. Since its concept, the model has been updated to include among other parts, a mix of hygroscopic molecules, called Natural Moisturizing factors (NMF). By binding water molecules, the role of NMF is to maintain the SC hydration at optimal levels that support both the water barrier function (2) and the SC elastic qualities (3). The NMF is composed primarily of free amino-acids (alanine, glycine, histidine, arginin+ornine+citrine, proline, serine), and their derivatives [glutamine-derived pyrrolidone carboxylique acid (PCA), and histidine-derived trans urocanic acid (tUCA)], as well free ions, sugars, urea, and lactate (4,5). These hygroscopic molecules represent 10% of the corneocytes mass (6) and 20 to 30% of the dry weight of the SC (2). The majority of the free amino acids are derived from the complete hydrolysis of filaggrin (FLG) (7) and FLG-like proteins, which occurs during cornification, a process describing the maturation, stiffening, and migration of corneocytes toward the more superior layers of the SC (8). Some events can impair the natural breakdown of the FLG such as UV irradiation (2), chronic skin occlusion and certain harsh skin care practices.

The SC water content contributes to the homeostasis of human skin (9) by controlling its viscoelastic properties and its barrier function. Evaluation of skin hydration can be achieved non-invasively either by electrical (capacitance, conductance, etc.) or by spectroscopic methods. Electrical methods are based on the principle that higher water content facilitates easier flow of electrical charge. Spectroscopic methods use direct information of energy absorption by water molecules and are therefore more specific

than electrical methods. Moreover, with Raman confocal microspectroscopy, in addition to the SC water content, we can obtain information relating to the SC molecular composition (10), as a function of depth inside the SC.

Skin properties can change due to chronological aging and/or photoaging. As a result of chronological (intrinsic) aging the skin surface appears pale and is characterized by fine wrinkles, non-uniform pigmentation and the tissue gradually loses its elasticity. Extrinsic aging (photoaging) is characterized by deeper wrinkles, dry and sallow appearance and with mottled pigmentation. The loss of elasticity is more pronounced in photoaging. Body site differences exist with regards to effects of intrinsic and extrinsic aging (11,12). For example, epidermal cell turnover rates decrease with aging more rapidly for the facial skin compared to other body sites (13).

Interestingly, the effects of both intrinsic and extrinsic aging on the skin moisturization are not completely clear and reports are contradicting. In this study we investigated the effects of aging, photoaging, and body location on SC water content and the NMF composition using non-invasive methods.

Materials and Methods

a) Population

The study was conducted in accordance with the ethical principles of The Declaration of Helsinki. Forty (40) healthy female volunteers with Fitzpatrick skin types I-III and with no dermatological conditions participated in the study following signed informed consent. The volunteers were selected to cover the age range 20-70 years. All measurements were performed following 15 min of acclimatization in an environmentally controlled room (20-25 °C, 40% relative humidity). They did not expose themselves intentionally for

a long time to the sun or tanning beds for at least one month before measurement and they have not used self-tanning products, which might interfere with the Raman measurements by introducing high background fluorescence. They were not allowed to use any skin care product or deodorant. Non-invasive measurements were performed on three skin sites: face (central cheek area), relatively exposed arm site (dorsal forearm) and relatively protected arm site (upper inner arm).

b) *In vivo* measurements

The quality of the skin's barrier function was evaluated measuring trans-epidermal water loss (TEWL) using a closed chamber instrument (Vapometer, Delfin, Kuopio, Finland) (14). The average of 6 measurements was recorded for each volunteer and each skin site.

Skin surface hydration was evaluated using high frequency skin conductance (Skicon-200EX, I.B.S. Company, Ltd., Japan) (15). This method measures electrical properties relating to the hydration level of the upper SC. The average of 6 measurements was recorded for each volunteer and each skin site.

SC thickness, total SC water content and NMF composition (amino acids and their derivatives) were evaluated using in vivo confocal Raman microspectroscopy (Skin Analyser model 3510, River Diagnostics, Rotterdam, The Netherlands). A 671-nm laser was used to collect data at the high wavenumber spectral range (2600-3800 cm^{-1}) for the determination of water content. A 785-nm laser was used to collect Raman spectra in the fingerprint region (400-1800 cm^{-1}) from which each amino acids, lactate and urea levels were determined. In the high wavenumber region spectra were taken at depths through the SC from 0 to 32 µm every 4 µm and in the fingerprint region from 0 to 24 µm depth every 4 µm. A total of 10 measurements were acquired in each wavenumber region at each site and for each volunteer. The Raman instrument was calibrated once at the beginning of each experiment day according to manufacturer's instructions. Before each measurement at a different skin site the CaF_2 window was cleaned up with a single

wipe of tissue with a drop of methanol.

c) Spectral analysis

To evaluate the water content inside the SC expressed in g water per 100 g wet tissue (mass %), we used the intensity ratio of water/protein as described by Caspers et al. (16) The SC thickness was calculated using the water profile concentration as described by Bielfeldt et al. (17) and Egawa et al. (18,19). The average SC thickness of the 10 measurements of the water profile for each skin site and each volunteer was calculated. Then, from these values the average SC thickness for each site and each age group was used to normalize the depth values (x-axis) for the calculated concentration profiles. The total amount of each component was calculated as the integrated value between the normalized depths 0 (surface) and 1 (junction between SC and *Stratum Granulosum*).

The concentrations of NMF components, including alanine, glycine, histidine, arginin+ornine+citrine, proline, serine, lactate, Urea, trans Urocanic acid, and Pyrrolidone Carboxylique Acid were calculated from Raman spectra in the region 400-1800 cm^{-1} using a previously published algorithm (10). The total Amino Acids was calculated as the sum of Alanine, Glycine, Histidine, Ornine, Proline, and Serine contents. The total NMF was calculated as the sum of Amino Acids, Lactate, Urea, Pyrrolidone Carboxylique Acid , and trans Urocanic acid contents.

d) Statistical analysis

Linear regression as a function of age was tested for each of the following parameters: total SC water, Alanine, Glycine, Histidine, Ornine, Proline, Serine, Lactate, Urea, trans Urocanic acid, Pyrrolidone Carboxylique Acid, Amino Acids, and total NMF. For each distribution the regression coefficient R^2 and the significance of the correlation p were

calculated. For each distribution mean +/- one standard error of mean are shown in the graphs. Statistical comparison of two distributions was performed following the Anderson-Darling normality test and test of variance (F-test) in order to select the appropriate t-test.

Statistical significance was accepted at the level of α=0.05.

RESULTS

In this study we investigated the effects of body site location as well as intrinsic and extrinsic aging on SC water content and NMF composition using non-invasive methods.

a) The SC water content and NMF composition dependents on body site location

Our results (Table 1) show that SC on the face contains significantly more water than the SC on the two arm sites tested (Figure 1). However, the total NMF and the total amino acid contents do not show significant differences between the three skin sites in each of the four groups, although the relative amount of each NMF component depends on body site location. The SC histidine and alanine contents are similar for the three skin sites regardless of age. Significant differences are expressed for the glycine and serine components depending on the site. The SC on the face appears to contain less glycine and serine than that of the arm regardless of age. In contrast, the SC lactate content is higher on the face compared to the two arm sites independent of age. Moreover, the SC proline content is higher on the face compared to the protected arm site only for the older groups tested (> 40 years of age). The SC contents for the amino-acid derivatives Pyrrolidone Carboxylique Acid and trans Urocanic acidare mostly equivalent between the sites except for the youngest group for which these parameters are higher for the face compared to the arm sites.

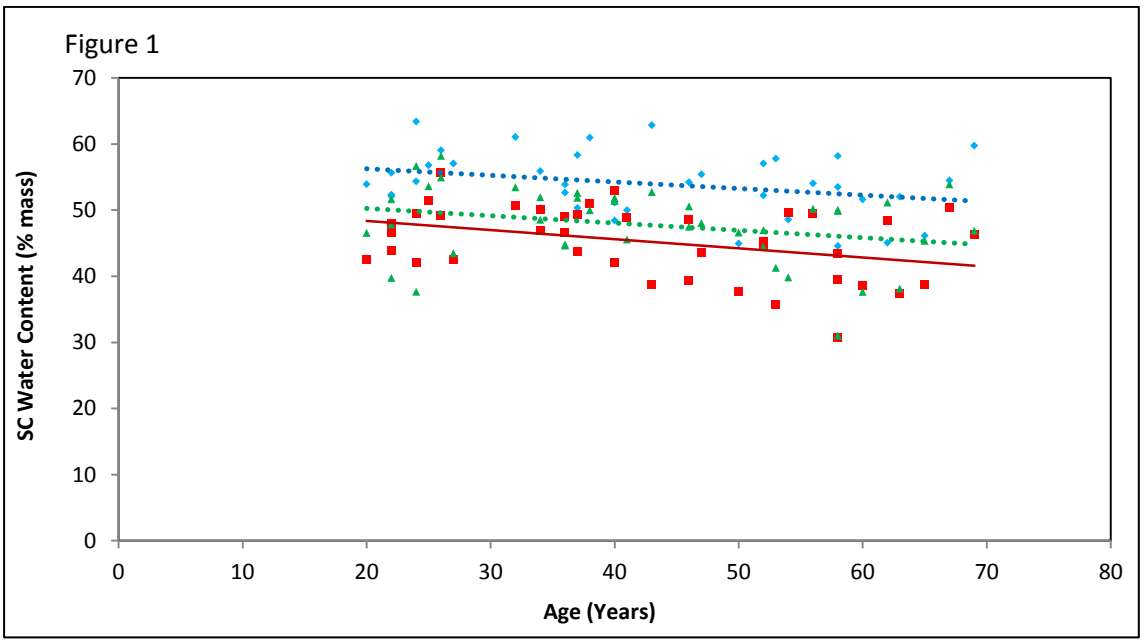

***Figure 1: SC water content depends on age and skin site**. Individual data points are shown for face (cheek, blue diamonds), dorsal forearm (red squares), and upper inner arm (green triangles). Significant correlation was found the dorsal forearm (y=-0.1381*x+51.13, R^2=0.14, p <0.001). No statistical difference was found for the face or the upper inner arm.*

Table 1 Summary table of the parameters measured and their dependence on age and skin site
With alanine (Ala), glycine (Gly), histidine (His), arginin+ornine+citrine (Orn), proline (Pro), serine (Ser), lactate (Lac), Urea, trans Urocanic acid (tUCA), and Pyrrolidone Carboxylique Acid (PCA), Amino Acids (AA) and Natural Moistuirizing Factors (NMF).

Method	Parameters	Age	Site (face vs arm)	Exposure(exposed vs protected arm site)
RAMAN	**[Total water]**	Decreases on exposed arm site	Higher on face	No difference
	[Total NMF]	Increases on face	No difference	No difference
	[Total AA]	Increases on face	No difference	No difference
	[Lactate]	Decreases on protected arm site and exposed arm site	Higher on face	No difference
	[Ala]	Increases on face and on exposed arm site	No difference	No difference
	[Gly]	Increases on protected arm site	Less on face	No difference
	[His]	_	No difference	No difference
	[Orn]	Decreases on protected arm site	No difference	No difference
	[Pro]	Increases on face and decreases on protected arm site	No difference except for the last two groups of age, higher on face	No difference
	[Ser]	Increases on protected arm site and exposed arm site	Less on face	No difference
	[Urea]	Decreases on protected arm site	No difference	No difference
	[tUca]	_	No difference except on face for the youngest group: less on face	No difference
	[PCA]	_	No difference except on face for the youngest group: less on face	No difference

b) The SC water content and NMF composition are affected by aging

Linear regression of the SC water content vs. age shows a general small decrease with advancing age on all skin sites tested (Figure 1). This decrease is significant for the exposed arm site. Linear regressions of the total NMF and total amino acid contents vs. age show a general increase for all sites tested (Figure 2). This increase reaches significance levels for the face. Age affects differently the various NMF components (Table 1). Notably, linear regression of the SC lactate content vs. age shows a general decrease that reaches significant levels for the two arm sites (Figure 3). Interestingly, the rates of change (regression slope) of the SC lactate content for the two arm sites are very close.

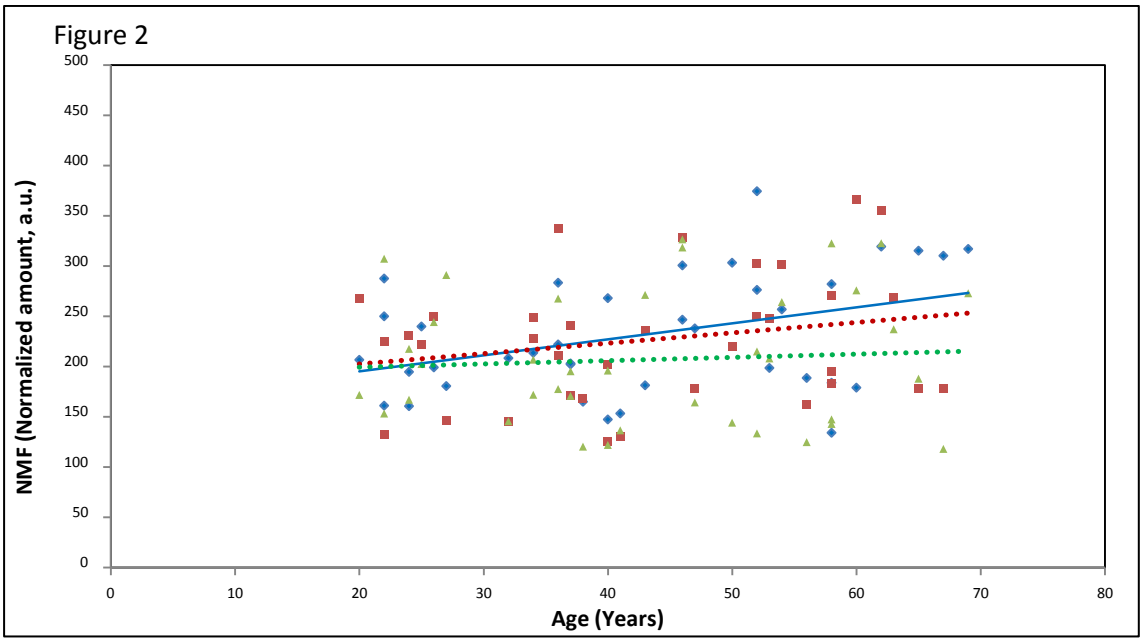

Figure 2: NMF concentration depends on age and skin site. *Individual data points are shown for face (cheek, blue diamonds), dorsal forearm (red squares), and upper inner arm (green triangles). Significant correlation was found the face (y=1.59*x+163.313, R^2=0.018, p <0.001). No statistical difference was found for the dorsal forearm or the upper inner arm.*

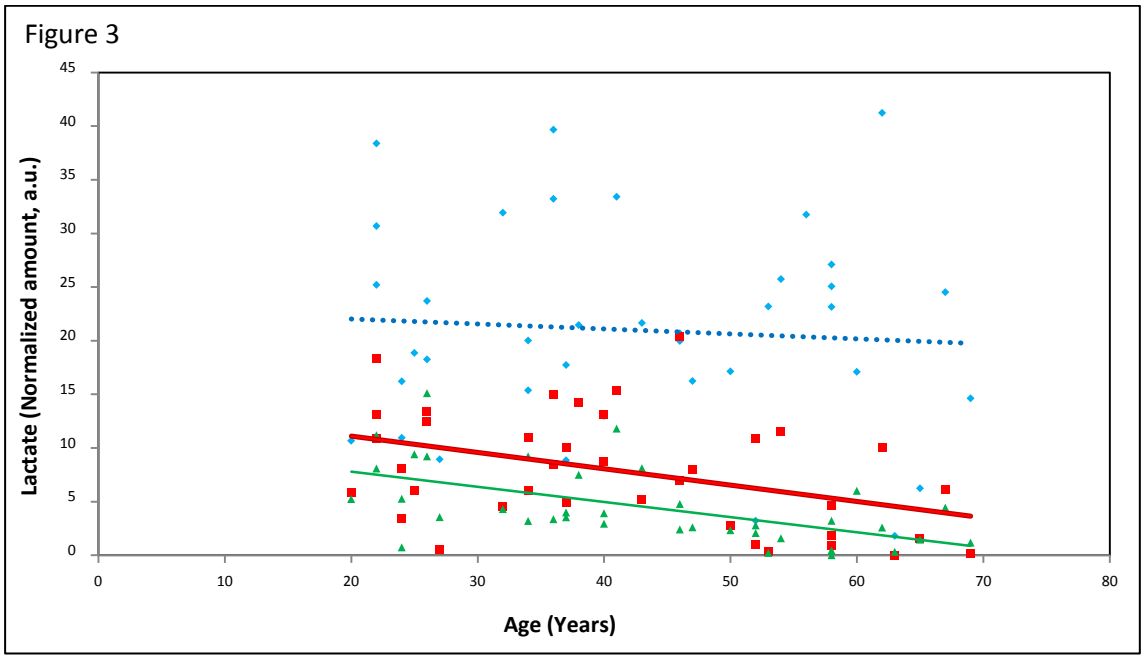

Figure 3: Lactate concentration depends on age and skin site. *Individual data points are shown for face (cheek, blue diamonds), dorsal forearm (red squares), and upper inner arm (green triangles). Significant correlation was found on upper inner arm and dorsal forearm: the upper inner arm (y=-0.136*x+10.11, R^2= 0.294, p <0.001) and the dorsal forearm (y=-0.153*x+14.17, R^2=0.169, p=0.001).*

Discussion

In this work we investigated the effect of age and body site location on the SC water content and the SC NMF composition using non-invasive methods in vivo. To that end, we conducted a study involving 40 European Caucasian women divided into four groups according to the age from 20 to 70 years. The investigation was done on three skin sites: face, exposed arm site, and protected arm site. Intrinsic and extrinsic skin aging effects are explored on the two selected arm sites. The importance of the skin on the face is that it is physiologically and structurally different from the two arm sites. Our results show that intrinsic and extrinsic skin aging affect SC hydration, while they have variable effects in the concentrations of NMF components depending on the skin site.

1. *Does the SC water content depend on skin site location?*

Our data show that the SC water content is higher on the face compared to the two arm sites in agreement with previous reports (19). Compared to other body sites the SC is thinner on the face and the epidermal cell turnover rate is higher on the cheek compared to the arm (12,13). Besides biological factors, cosmetic habits (e.g. women are used to apply more moisturizing products on the face) may also explain the differences we see in SC water content between the face and the two arm sites. Comparison of the SC water content between the two arms shows no statistically difference regardless of age indicating negligible effects of photoaging for this parameter.

2. *Does the SC biochemical composition depend on skin site location?*

The health of the SC depends on the maintenance of the SC water content at an optimal level. The biochemical composition of the SC, including the NMF, plays an important role for the maintenance of the SC hydration. Our results show that the total NMF content and the total amino acids do not vary significantly between the three skin sites tested. Most NMF components derive from the degradation of FLG by proteolysis (7) during the cornification process (5,20). These hygroscopic molecules are exclusively found in the SC (21). Interestingly, the SC content for different amino acids and their derivatives PCA and UCA is not the same between the three sites tested. This could imply that besides FLG there are other SC proteins (such as hornerin (HRNR) and caspase-14) that can be hydrolyzed and act as amino acid sources. HRNR contributes to skin moisturization by forming NMF in a similar way as proposed for FLG (21),(22) and is detected at the corneocyte periphery in the entire cornified layer (23). It can be hypothesized that

proteins like HRNR may be expressed at different levels depending on the location (23). Furthermore, our results show that the SC lactate content is higher on the face compared to the arm sites. Lactate is known to have a moisturizing effect (24) which could explain why the skin on the face is more hydrated than on the two arm sites (12). Lactate may arise from anaerobic metabolism of highly proliferating cells such as those in the basal layer of the epidermis and can rapidly diffuse to the SC (25). Thus the higher epidermal turnover rate on the face may explain the higher lactate content.

3. *How does photoaging affect the SC water content?*

Our data show that the SC water content significantly decreases as a function of age on the exposed arm site, although the overall decrease is small (about 10%). There was no significant decrease of the SC water content for the face and the protected arm site indicating that chronological aging has minimal impact on skin dryness. This finding is in general agreement with Egawa and Tagami (19) for the face (cheek) site. The same authors find a small decrease for the forearm site (presumably the volar forearm, although it is not specified in their publication) in agreement with our results for the dorsal forearm (exposed). It is possible that a slower cell turnover rate on the arms compared to the face, can lead to an accumulation of the amino acid content, which can explain the site differences we observed.

4. *How does the age affect the SC biochemical composition?*

Our data show that globally the total NMF content and the total amino acids content significantly increase with age on the face. On the other hand, the total amino acids

content does not change significantly with aging on either arm site, indicating that the photoaging does not significantly affect amino acid production in the SC. This finding may be surprising as previous reports have shown that UV exposure disrupts enzymatic breakdown of FLG (26) which would be expected to result in a decrease in the concentration at least of some amino acids on the exposed arm site. However, we have previously shown that: 1) epidermal turnover rates decrease with age and 2) SC thickness increases with age. This can explain why aging can lead to accumulation of amino acids in the SC.

From the NMF components that we could detect with Raman, lactate content significantly decreases on both arm sites. Lactate content is known to correlate with the SC hydration state (24). Lactate represents 10% of the total NMF (24). It is supposed that lactate mainly originates from sources outside the SC such as from sweat (24). As mentioned above another source of lactate is from anaerobic metabolism of the proliferating keratinocytes at the basal layer of the epidermis. Being a small molecule, lactate can rapidly diffuse from the basal layer to the SC. As the rate of change of lactate content with age is similar for both arm sites, it seems that chronic environmental exposure plays a minor effect on this decrease.

In conclusion our results show that both intrinsic and extrinsic aging have an impact on the SC water content and the SC biochemical composition. They both affect the SC hydration by leading to variable effects in the concentration of the NMF components inside the SC.

Conflict of interest

The work was fully founded by Johnson & Johnson Santé Beauté France. GNS is an employee of this company.

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III ARTICLE 4

Mobility of Water Molecules in the Stratum Corneum: Effects of Age and Chronic Exposure to the Environment

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Key Words: Hydrogen bond, Raman Spectroscopy, Skin Barrier, Natural moisturization factors

Short title: Water mobility in the stratum corneum

Abbreviations: NMF, Natural Moisturization Factors; SC, stratum corneum

To the editor:

It is well established that water in the stratum corneum (SC) plays a critical role in skin physiology (Rawlings and Matts, 2005). Depending on their mobility, determined by the strength of hydrogen bonds and space limitations, SC water molecules can be grouped into three categories (Visscher et al. n.d.; Bulgin and Vinson 1967; Walkley 1972; Takenouchi et al. 1986; Walling and Dabney 1989; Gilard et al. 1998; Gniadecka et al. 1998; Kasting et al. 2003; Pieper et al. 2003; Yadav et al. 2007; Nakagawa et al. 2011; Vyumvuhore et al. 2013): a) “bound” (least mobile), molecules directly hydrogen-bonded to SC structural molecules; b) intermediately mobile, molecules that form hydrogen bonds with “bound” water molecules and can be secondary, tertiary or higher order, forming a “loose cloud”around the binding site; and c) most mobile, molecules that can diffuse more freely, continuously forming and breaking hydrogen bonds with their surrounding water molecules.These “states” are weakly defined and in reality there is a continuum of bound states. However, it can be useful to employ this analysis as means of providing some perspective to the mobility of water in the SC.

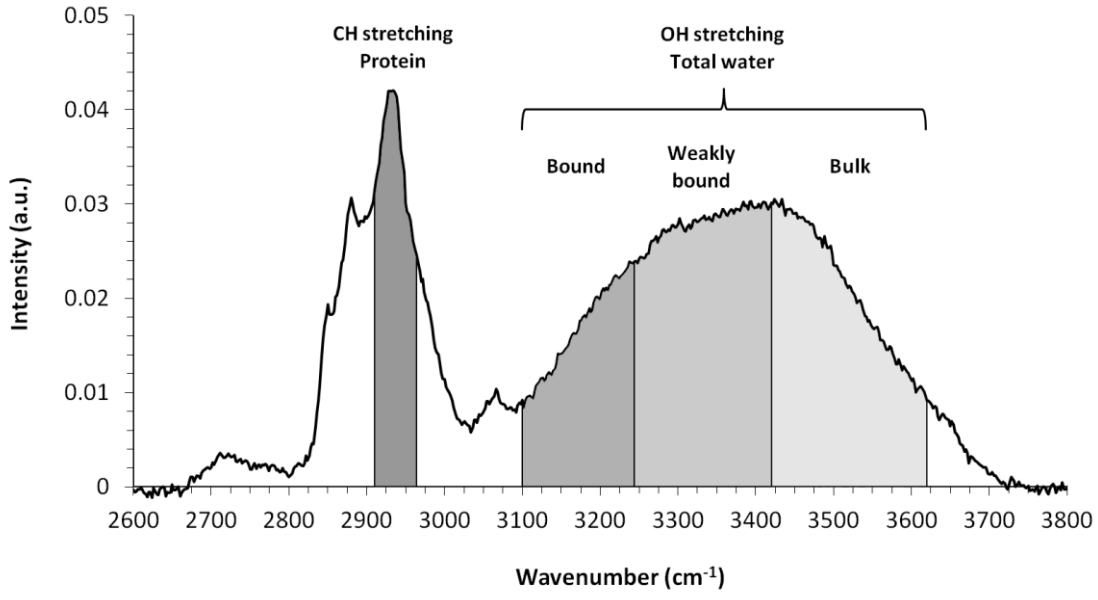
Confocal Raman microspectroscopy is the only *in vivo* method to date that can generate concentration profiles of SC components as a function of distance from the skin surface (Caspers et al. 2001). Water concentration profiles (% mg water/mg protein) can be constructed following a calibration procedure, using the spectral bands 3350-3550 cm^{-1} (OH stretching) and 2910-2965 cm^{-1} (CH stretching). Water content is low at the skin surface and gradually increases to a plateau at the junction between the SC and the stratum granulosum. However, the range 3350-3550 cm^{-1} is limited to the spectral contributions of the more mobile water molecules (Vyumvuhore et al. 2013). These authors have defined the band ranges (sub-bands of the OH stretching mode) that correspond to the three mobility states described above.

The objective of the current study was to examine if age and chronic environmental exposure affect the relative distribution profiles of these three states in the SC. The production of natural moisturization factors (NMF), that may influence these

distribution profiles and their dependence on age and exposure to the environment are also examined.

The study was conducted according to the principles of The Declaration of Helsinki. Healthy female volunteers participated in the study following written informed consent and were divided into two age groups of 10 volunteers in each: 20-30 and 50+ years of age. Raman data (Skin Analyzer 3510, River Diagnostics, Rotterdam, The Netherlands) were acquired on three skin sites: face (cheek), relatively exposed arm site (dorsal forearm) and relatively protected arm site (upper inner arm), following 15 min acclimatization in an environmentally controlled room (20 °C, 40% relative humidity). The concentration profiles of water and total NMF, as well as the areas under the curve for the spectral bands defined by Vyumvuhore et al. (**Figure 1a**) were calculated from the Raman spectra using the Skin Tools software (River Diagnostics). To account for interpersonal differences in the SC thickness, the distance from the SC surface was normalized to the SC thickness measured from the Raman data (Bielfeldt et al. 2009). Data are presented as mean +/- one standard deviation. For group comparisons a Student's t-test was performed following confirmation of normality (Anderson-Darling test) and statistical significance wasaccepted at the level of α=0.05.

1.a



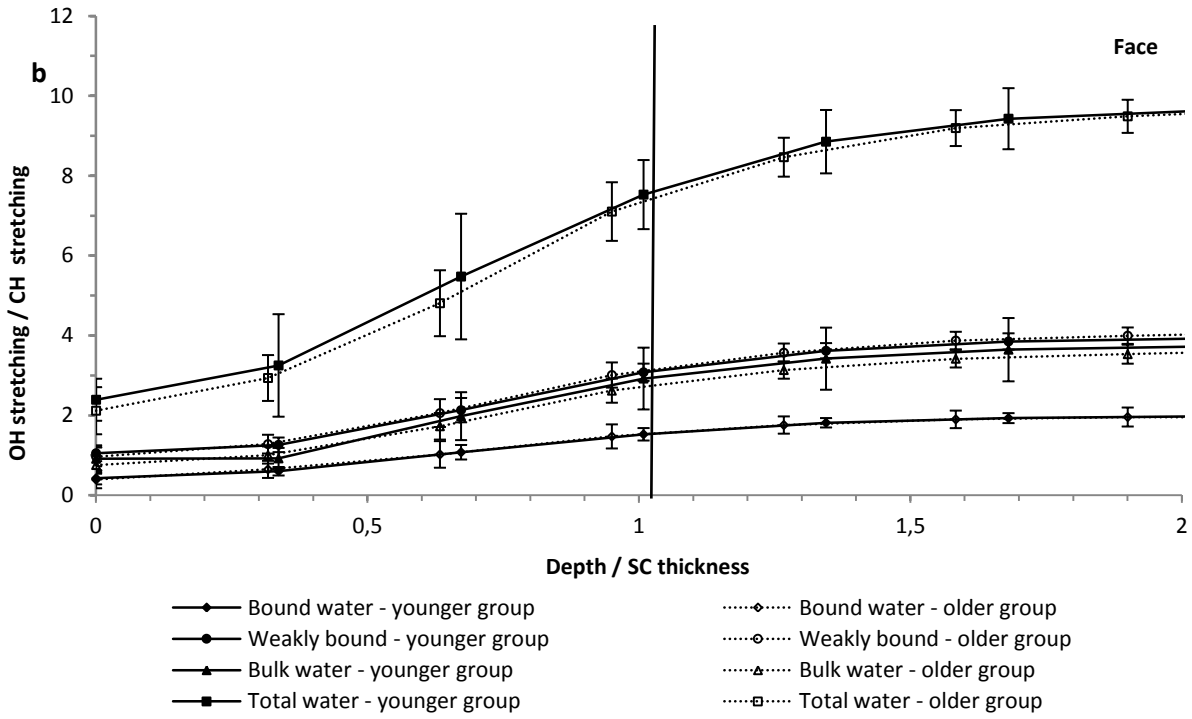


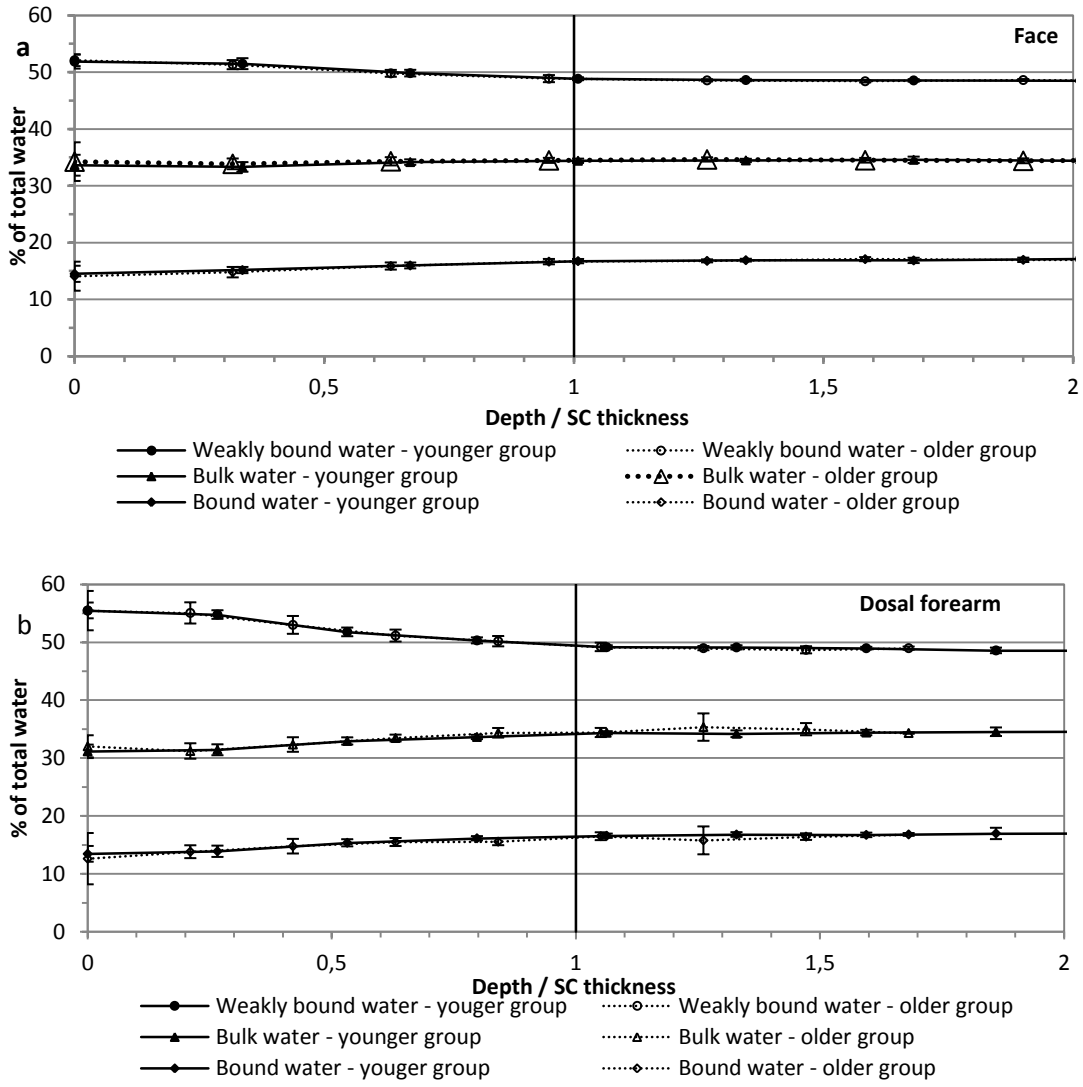
Figure 1. The mobility of water molecules in the stratum corneum can be studied in vivo. *(a) Raman confocal spectra in the high wavenumber region highlighting the bands of CH stretching and OH stretching that can be used for the calculation of water content and that of the water molecules in the three states based on their mobility. (b) The depth profiles of the three states of water according to molecular mobility. The OH stretching bands have been normalized to the CH stretching. There are minor differences between the two age groups studied. Data are presented as mean +/- one standard deviation.*

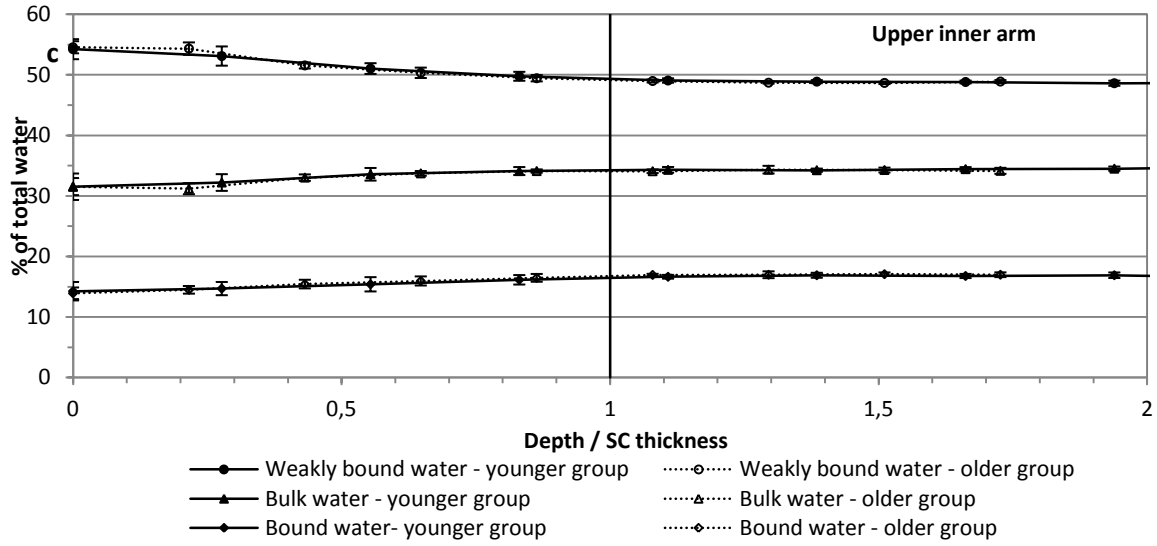
The water concentration profiles for the three water states show qualitatively similar pattern with that of total water: a low value close to the skin surface that monotonically increases to a plateau at the base of the SC (**Figure 1b**). There are minor differences between the two age groups, but distinct differences between body sites: the SC of the face has higher total water content compared to the arm sites.

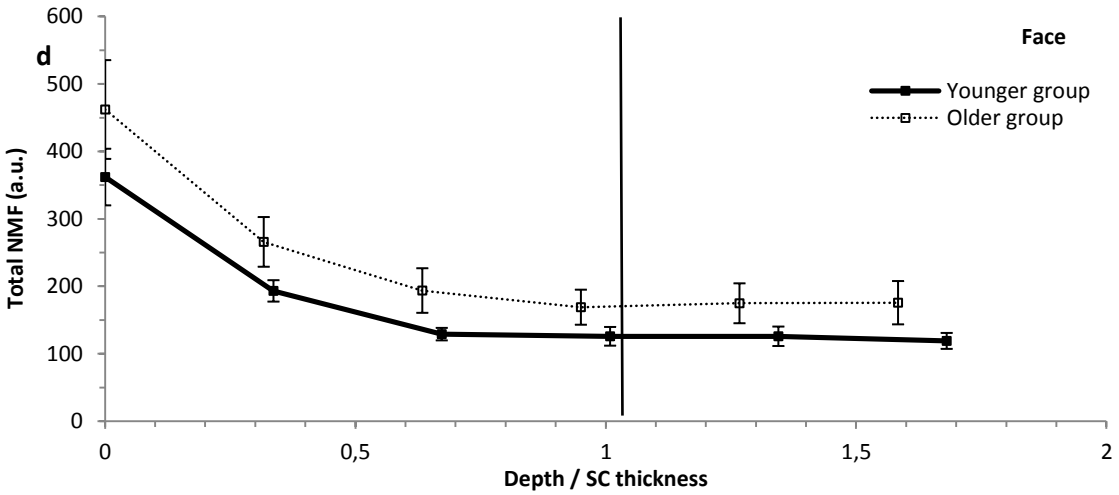
Normalizing the profiles of the three water states to the total water we get a different pattern that appears to be highly conserved between the two age groups and the three body sites tested (**Figure 2a-c**). While the normalized profiles of the bound and

most mobile water molecules decrease towards the skin surface, that of the intermediate group increases. The changes are subtle (<6% difference), but statistically significant and with low interpersonal variability (<7%). Interestingly, in the viable epidermis the relative percentage of the three groups appears to be constant, independent of age or body site. The molecular ratios of bound :intermediate: most mobile water are 49:34:17.

Figure 2. The distributions of the three states of water according to molecular mobility are independent of age and skin site. *Data for the two age groups are shown for (a) the face, (b) the dorsal forearm, and (c) the upper inner arm site. (d) The total Natural Moisturization Factor profile expressed as the sum of the NMF components as a function of depth from the surface. Note the increased NMF towards the skin surface following protein (e.g. filaggrin) break-down processes. All data are presented as mean +/- one standard deviation.*







Throughout the SC, corneocytes migrate and mature towards the surface (Harding et al. 2003), while proteins of the S100 family, including filaggrin, filaggrin-2, and hornerin, are broken down to give rise to an assembly of amino acids and other products, known as NMF (Rawlings 2010). The sum of the profiles of these molecules is used to calculate the total NMF concentration profile (**Figure 2d**). The gradual increase of the total NMF

concentration towards the SC surface is indicative of the proteolytic processes along the SC exposing pockets of bound water molecules, increasing their mobility. This may explain the decrease in the relative amount of bound water and the concurrent increase in the amount of intermediately mobile water towards the SC surface. Another possible explanation is that part of the bound water is linked to lipid head-groups (Yadav et al. 2007) and the relative amount of this state is decreased as the lipid content in the outer SC is depleted. SC equilibrated to ambient conditions of 20-40% relative humidity is expected to have lower water content as we move towards the skin surface and this water is expected to be more tightly bound pointing to the humectant role of the NMF. This could explain the shiftfrom the most mobile water molecules towards the intermediately bound.

These observations provide important baseline information for the mobility state of water in the SC in healthy skin. This can be of interest inthe study of water diffusion through the SC and trans-cutaneous drug delivery. The surprising consistency of the profiles between age groups and body sites imply that there is a tightly controlled mechanism that defines them. Such mechanisms are expected to be affected by the environmental temperature and humidity conditions (Vyumvuhore et al. 2013). Further studies will shed light on whether these patterns are conserved or differ in the case of pathological skin conditions, including barrier-related disorders, such as psoriasis, atopic dermatitis, icthyosis, senile xerosis, diabetic-related dry skin etc.

In conclusion, chronological aging and chronic exposure to environmental factors do not affect the normalized concentration profiles of the three mobility states of water in the SC. While the profiles are flat in the viable epidermis, they demonstrate slight changes towards the SC surface. These changes are consistent between the age groups and body sites tested and they can be explained by the enzymatic proteolysis of filaggrin and similar molecules.

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CONCLUSION GENERALE

|CONCLUSION GÉNÉRALE

Les travaux de cette thèse ont couvert différents aspects concernant l'impact du vieillissement cutané sur le *Stratum Corneum* par des méthodes non invasives aux niveaux tissulaire, moléculaire et supra-moléculaire *in vivo*. Plusieurs méthodes analytiques ont été employées telles que des méthodes vibrationnelles spectroscopiques : la micro-spectroscopie confocale Raman et la spectroscopie ATR-FTIR, ainsi qu'une méthode séparative : la chromatographie liquide en phase normale couplée à un spectromètre de masse haute résolution dotée d'une source APCI et d'un détecteur orbitrap (NP-LC-HR-MS). Ces dernières années, de nombreux descripteurs spectroscopiques ont été exploités permettant ainsi une étude fine de la physiologie cutanée. Mon travail de thèse a utilisé ces descripteurs afin d'étudier l'évolution de la fonction barrière cutanée et la fonction barrière hydrique lors du vieillissement cutané sur des femmes caucasiennes âgées de 18 à 75 ans.

La méthode spectroscopique principalement utilisée ici est la micro-spectroscopie confocale Raman. Elle a été mise en lumière en 1920 par M. Raman et est aujourd'hui en plein essor. Elle est très bien décrite dans la littérature existante. Cette méthode performante et fiable est utilisée dans les études *in vivo* du fait de son caractère non-invasif. Elle permet d'obtenir des informations tissulaires, cellulaires, moléculaires et supra-moléculaires sur chacun des constituants du SC en profondeur dans l'épiderme. Cette méthode non destructive permet donc de garder le contexte physiologique ainsi que la réactivité physiologique des constituants et notamment l'eau dans différentes conditions. Ainsi, il est nécessaire d'utiliser cette technique pour étudier l'hydratation *in vivo* dans la couche cornée, la cornéométrie ne suffisant pas. Elle permet ainsi de mesurer les profils de concentration des composés du SC avec précision. Cette technique permet aussi de caractériser *in vivo* l'organisation des chaînes lipidiques. Une réflexion approfondie est nécessaire afin d'interpréter les spectres et expliquer les résultats. Cette technique vibrationnelle est complémentaire de la spectroscopie infrarouge et les deux combinées permettent ainsi d'avoir la signature vibrationnelle totale du SC.

Le vieillissement naturel ou induit par les agressions extérieures est un sujet qui a soulevé beaucoup d'interrogations et d'intérêt. De nombreuses publications relatent les effets du

vieillissement principalement au niveau du derme et peu de publications explicitent les variations physiologiques au niveau de l'épiderme. La première partie de ce travail de thèse a consisté à dresser un constat à partir de la littérature existante, des connaissances de l'évolution de la couche cornée avec le vieillissement chronologique et photo-vieillissement au niveau épidermique. Un chapitre de livre a été rédigé en se focalisant en particulier sur les changements relatifs au SC et les méthodes d'investigation *in vivo*.

Ce constat établi, plusieurs aspects de la barrière cutanée ont fait l'objet de travaux expérimentaux. La barrière cutanée est caractérisée à la fois par un état de compacité des édifices lipidiques et un état d'hydratation cutanée. L'équilibre subtil entre les deux confère la fonction barrière.

La première partie expérimentale *in vivo* concerne l'évolution de la fonction barrière avec l'âge. Celle-ci a été réalisée sur un groupe de femmes caucasiennes et a montré que l'intégrité de la fonction barrière était conservée lors du vieillissement chronologique et du photo-vieillissement. Le vieillissement chronologique semble avoir un impact tissulaire du fait de l'augmentation du SC alors que le photo-vieillissement a un impact moléculaire et supramoléculaire sur l'organisation des édifices lipidiques intercornéocytaires. Ainsi, l'augmentation de l'épaisseur de la couche cornée compense la diminution du taux de lipides ainsi que l'affaiblissement de la compacité du ciment lipidique avec l'âge. Quelque soit l'âge, les lipides des couches les plus superficielles du SC sont organisés dans une phase orthorhombique. Les variations limitées de la micro-hétérogénéité des céramides du ciment lipidique comme l'augmentation du rapport H-CER/P-CER, la variation des longueurs de chaînes ou le nombre d'insaturations des chaînes carbonées peut expliquer les faibles variations observées lors du vieillissement. L'ensemble de ces résultats prouve le maintien de la fonction barrière cutanée avec l'âge chez les peaux saines. Il est également intéressant de constater que la joue possède une fonction barrière plus faible que celui du bras exposé et du bras protégé. Son comportement physiologique cutané est différent du reste du corps, mais le SC de la joue évolue de la même façon que celui des autres sites corporels. Avec cette étude, il est clair que, seule, la mesure globale et classique de PIE n'est pas suffisante pour caractériser la fonction barrière cutanée.

Dans un deuxième temps, nous nous sommes intéressés à l'évolution de l'état d'hydratation de la couche cornée lors du vieillissement cutané car état barrière et état d'hydratation cutané sont intimement très liés. La barrière cutanée influence l'état d'hydratation par sa gestion des flux hydriques et la présence des NMF dans le SC alors que l'état d'hydratation influence la compacité des édifices lipidiques à l'origine de la qualité de la fonction barrière cutanée par la présence des liaisons hydrogènes au sein de ces édifices lipidiques. Bien que la conductance méthode globale de mesure de l'hydratation soit peu sensible à ses variations, notre étude a montré que le vieillissement cutané avait peu d'impact sur l'état d'hydratation qui participe au maintien de l'homéostasie cutanée. L'augmentation de l'épaisseur du SC en vieillissant ainsi que le ralentissement du taux de desquamation et une légère augmentation de la teneur en NMF compensent la diminution de la production des lipides du SC. Cependant, des variations moléculaires dans le groupe des NMF existent.

Le pourcentage par rapport à l'eau totale de chacun des trois états de mobilité de l'eau: eau partiellement liée, eau libre et eau non-liée semble très stable avec le temps. Les changements mineurs observés peuvent être en partie expliqués par des variations de processus enzymatiques à l'origine de la desquamation ou de la production de NMF. Là encore, le SC de la joue, physiologiquement différent, se comporte de façon similaire aux SC des deux autres sites corporels du bras exposé et du bras protégé. L'âge n'est donc définitivement pas un facteur critique pour le maintien de la fonction barrière hydrique chez les femmes caucasiennes âgées de 18 à 75 ans ayant une peau saine et le photo-vieillissement n'amplifie pas les phénomènes de variations physiologiques.

Mon travail de thèse a permis d'évaluer l'impact du vieillissement cutané sur des femmes caucasiennes âgées de 18 à 75 ans à l'aide d'une méthodologie multimodale efficace pour avoir des informations aux niveaux moléculaire et supra-moléculaire. La fonction barrière résultant de la composition des édifices lipidiques et de l'état d'hydratation de la SC est maintenue avec l'âge pour la régulation de l'homéostasie cutanée. Il serait sans doute très intéressant de continuer ces études sur une population beaucoup plus âgée. Elles sont d'autant plus justifiées que la durée de vie s'est considérablement allongée.

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La peau est l'organe le plus étendu du corps humain. Doté d'une membrane biologique fine appelée la couche cornée, celle-ci le protège du desséchement et des agressions extérieures chimiques ou mécaniques auxquelles le corps humain doit faire face. Ce travail de thèse a consisté, dans un premier temps, à décrire via la littérature existante les effets de l'âge,dûs au vieillissement intrinsèque et extrinsèque,sur la physiologie cutanée du *Stratum Corneum* (SC). La partie expérimentale basée sur la microscopie vibrationnelle traitera des variations de la fonction barrière et de l'hydratation du SC lors du vieillissement chronologique et photo-vieillissement. D'autres méthodes ont également été utilisées comme la chromatographie liquide en phase normale couplée à la spectrométrie de masse haute résolution dotée d'une source APCI et d'un détecteur Orbitrap pour l'étude de la composition détaillée des lipides du SC ainsi que des méthodes plus globales comme la PIE ou la cornéométrie. Le caractère non invasif de toutes ces méthodes a permis de réaliser ces études *in vivo.* L'évolution de la fonction barrière a été étudiée aux niveaux tissulaire, moléculaire et supramoléculaire par micro-spectroscopie confocale Raman et spectroscopie infrarouge. Puis le lien moléculaire a été fait entre le vieillissement intrinsèque et les céramides de la matrice lipidique intercornéocytaire par Chromatographie en phase Liquide couplée à la Spectrométrie de Masse. Les molécules discriminantes entre population jeune et âgée ont été déterminées par analyse chimiométrique. L'évolution de l'hydratation cutanée aux niveaux tissulaire, moléculaire et supramoléculaire a également été l'objet d'une investigation approfondie. Les variations de la composition des NMF et la teneur en eau dans le SC lors du vieillissement cutané ont été mises en lumière en utilisant des descripteurs spectraux Raman. Les variations structurelles des molécules d'eau impactant l'organisation supramoléculaire des édifices lipidiques ont également été évaluées. Au cours du vieillissement, la fonction barrière cutanée et hydratation sont conservées.

Mots clés : Vieillissement chronologique et photo-vieillissement,fonction barrière, hydratation cutanée, SC, lipides, céramides, mobilité de l'eau, spectroscopie vibrationnelle (micro-spectroscopie confocale Raman et ATR-FTIR) et chromatographie liquide couplée à la spectrométrie de masse